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Departamento de Química

Joana Sofia Marques Pereira

Eco-engenharia de culturas microbianas produtoras de bioplásticos

Bioplastics production through mixed microbial cultures eco-engineering

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Dissertation submitted to the University of Aveiro to meet the requirements for the Degree of Master Biotechnology, performed under the scientific guidance of Prof. Luísa Serafim, Assistant Professor at Department of Chemisty, University of Aveiro, and Prof. Ana Xavier, Assistant Professor at Department of Chemisty, University of Aveiro.

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palavras-chave

Polihidroxialcanoatos, Culturas Microbianas Mistas, Processo em três fases, Alimentação Aeróbia Dinâmica, Reator Descontínuo Sequencial, Licor de Cozimento ao Sulfito Ácido

resumo

Os bioplásticos têm sido foco de interesse como alternativa sustentável aos plásticos convencionais. Entre os vários biopolímeros destacam-se os polihidroxialcanoatos (PHA), não só pela sua biocompatibilidade e biodegradabilidade, mas também porque podem ser produzidos por culturas microbianas mistas (MMC) a partir de resíduos agroindustriais. Desta forma é possível reduzir substancialmente o preço de produção destes polímeros e valorizar substratos alternativos. Os PHA apresentam características muito variadas de acordo com a sua composição, o que permite que sejam utilizados em diversas aplicações. As características do polímero podem ser manipuladas através do controlo de vários parâmetros operacionais durante o processo de produção.

A produção de PHA por MMC neste trabalho foi feita com recurso a um processo em três fases: acidificação de um subproduto da indústria papeleira, o licor de cozimento ao sulfito ácido acidificado (HSSL), seleção de uma cultura microbiana acumuladora de PHA e produção de PHA.

A seleção ocorreu num reator descontínuo sequencial (SBR), operado durante 180 dias, e cujas condições foram alteradas de forma a selecionar uma cultura acumuladora de PHA e com boa produtividade volumétrica de PHA. Três estados pseudo-estacionários (PSS) foram atingidos após sucessivos aumentos na pressão seletiva, uma indicação clara de que a MMC foi capaz de se adaptar ao substrato e às condições impostas.

No último passo do trabalho foram realizados vários testes de acumulação que permitiram validar a utilização de HSSL acidificado em condições diferentes e Condensado (outro subproduto da índustria papeleira) como substratos para a produção de PHA. O melhor teste realizado apresentou uma acumulação máxima de 74.4% cdw e uma produtividade volumétrica de 0.27 gPHA/L.h.

Este trabalho permitiu mostrar a potencialidade do uso de MMC produtoras de PHA como forma de valorização de subprodutos e resíduos agroindustriais.

keywords

Polyhydroxyalkanoates, Mixed Microbial Culture, Three-stage Process, Aerobic Dynamic Feeding, Sequence Batch Reactor, Hardwood Spent Sulphite Liquor

abstract

Bioplastics have been the focus of interest as a sustainable alternative to conventional plastics. Among those, polyhydroxyalkanoates (PHA) can be highlighted, not only for their biocompatibility and biodegradability, but also because they can be produced by mixed microbial cultures (MMC) from agro-industrial wastes. This allows to substantially reduce the production costs and valorize alternative substrates. PHA have a wide range of characteristics according to their composition, which allows them to be used in many applications. The polymers characteristics can be manipulated through the control of several operational parameters during the production process.

Production of PHA by MMC in this work was based in a three-stage process: acidification of a by-product of the paper industry, hardwood spent sulphite liquor (HSSL), selection of a PHA accumulating microbial culture and PHA production.

The selection step occurred in a sequencing batch reactor (SBR), operated for 180 days, and whose conditions were changed in order to select for a PHA-accumulating culture and with good PHA volumetric production. Three pseudo-stationary states (PSS) were achieved after successive increases in the selective pressure, a clear indication that the MMC was able to adapt to the substrate and to the imposed conditions.

In the last step of this work several accumulation assays were performed that allowed for the validation of the use of HSSL acidified under different conditions and Condensate (another byproduct of the paper industry) for PHA production. The best test performed achieved a maximum accumulation of 74.7% cdw and a volumetric productivity of 0.27 gPHA/L.h.

This work allowed to show the potential of the use of PHA producing MMC as a way of valorization of agroindustrial byproducts and residues.

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ABBREVIATIONS

-qAcet	Acetate consumption rate
-qBut	Butyrate consumption rate
-qProp	Propionate consumption rate
3H2MB	3-hidroxy-2-metilbutyrate
3H2MV	3-hidroxy-2-metilvalerate
3HA	3-hydroxyalkanoate
3HB	3-hydroxybutirate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
Acet	Acetate
ADF	Aerobic Dynamic Feeding
AN/AE	Anaerobic/Aerobic Process
AnD	Anaerobic Digestion
ATP	Adenosine Triphosphate
But	Butyrate
cdw	Cell Dry Weight
CoA	Coenzyme A
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen Concentration
EBPR	Enhanced Biological Phosphorus Removal
EPS	Extracellular Polymeric Substance
FF	Feast and Famine
GAOs	Glycogen-accumulating organisms
GC	Gas Chromatography
HA	Hydroxyalkanoate
HB	Hydroxybutyrate
HPLC	High Pressure Liquid Chromatography
HRT	Hydraulic Retention Time
HSSL	Hardwood Spent Sulfite Liquor
HV	Hydroxyvalerate
lcl-PHA	Long-chain-length PHA
LDPE	Low Density Polyethylen
LS	Lignosulphonates
mcl-PHA	Medium-chain-length PHA
MMC	Mixed Microbial Cultures
NADH	Nicotinamide Adenine Dinucleotide
OLR	Organic Loading Rate
OUR	Oxygen Uptake Rate

P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-co-3HV)	Poly(3-hidroxybutyrate-co-3-hidroxyvalerate)
P(3HV)	Poly(3-hydroxyvalerate)
PAOs	Polyphosphate Accumulating Organisms
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PLA	Poly(lactic acid)
PP	Polypropylene
ProdSp	Specific Productivity
ProdVol	Volumetric Productivity
Prop	Propionate
PS	Polystyrene
PVC	Polyvinyl chloride
qPHA	PHA production rate
qPHB	PHB production rate
qPHV	PHV production rate
SBR	Sequenced Batch Reactor
scl-PHA	Short-chain-length PHA
SCOAs	Short-chain Organic Acids
SRT	Sludge Retention Time
TCA	Tricarboxylic Acid
TSS	Total Suspended Solids
Val	Valerate
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plants
YPHAs/S	PHA production yield based on substrate

CHAPTER 1: INTRODUCTION

Plastics play a major role in today's society due to their numerous applications such as packaging materials, clothing, construction, components in electronics, or automobiles (Chen 2009). However, the extensive use of this material creates serious social and environmental problems related with their disposal. Conventional plastics are non-degradable, a characteristic that is linked to their unusual bonds and/or halogen substitutions, vast number of aromatic rings and high molecular weights (Alexander 1981). For this reason, plastics can remain for centuries in the environment, which represents a threat to both biotic and abiotic components of ecosystems (Kumar et al. 2015; Goldberg 1997).

Nowadays, solutions for plastic wastes include disposal at landfill locations, incineration and recycling (Ray & Bousmina 2005), but none of them presents itself as a suitable response for the problem. Landfills are inadequate due to fast growth of human population and consequent plastic garbage production (Suriyamongkol et al. 2007). Incineration implicates the formation of carbon dioxide and other gases that contribute towards global warming and pollution. Recycling plastic could be a sustainable solution but it causes changes in the material properties to a large extent, resulting in plastics of inferior quality (Suriyamongkol et al. 2007; Kumar et al. 2015).

As a response to this problem and to the growing global environmental concerns, attention was focused on bio-based polymeric materials. These bioplastics are biodegradable and eco-friendly, characteristics that promote their development and potentiate research in this field (Luengo et al. 2003a). According to a report by Allied Market Research, published in November 2015, global market for bioplastics is on track to reach $30.8 billion by 2020, registering a compound annual growth rate of 17.5% during 2015-2020. It also states that the rising environmental awareness among the consumers and substantial curiosity of the industry about the possibilities offered by biodegradability are the key factors responsible for the increasing adoption of bioplastics (Shukla 2015).

Polyhydroxyalkanoates (PHA) are bioplastics that stand out not only because they are biodegradable and biocompatible but also because they can be produced from agro-industrial wastes. This makes PHA suitable to be inserted in a biorefinary focused in the valorization of these residues and by-products, as proposed in this work.

1.1. OBJECTIVES

This work aims to:

- Study the production of PHA by mixed microbial cultures (MMC), using a by-product of the paper mill industry;
- Select a PHA producing consortium of microorganisms with an aerobic dynamic feeding strategy using acidified high spent sulphite liquor (HSSL) as substrate;
- Optimize selection conditions in order to obtain a culture with high PHA accumulation rates and volumetric productivity;
- Optimize accumulation conditions in order to maximize PHA content, yields and productivities;

An overview of the work developed is illustrated in Figure 1.

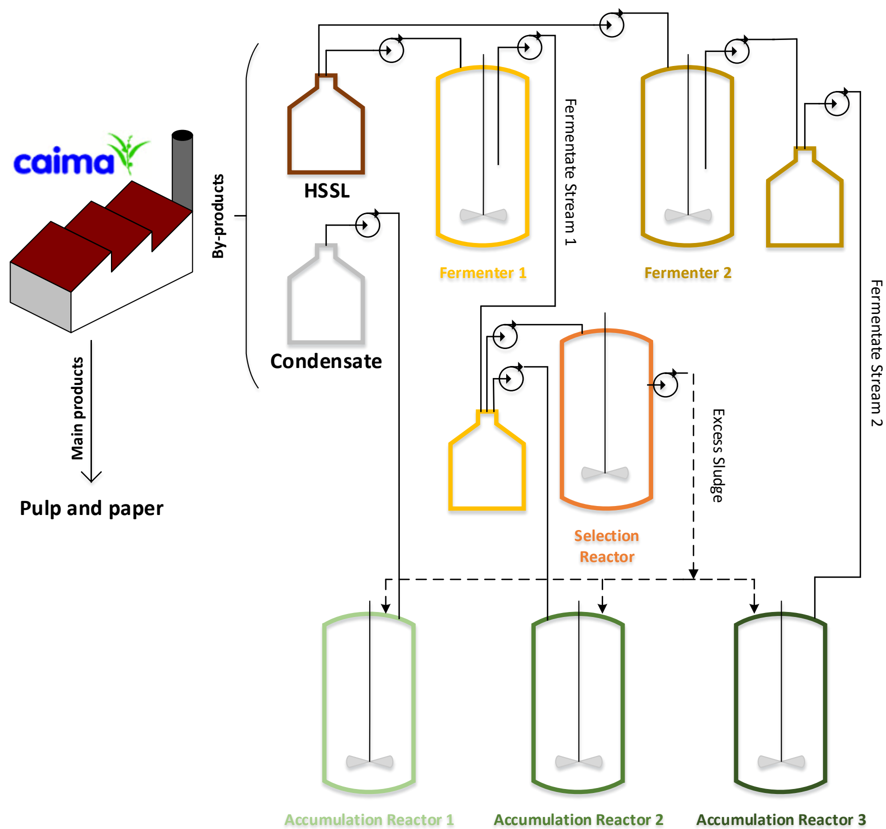


FIGURE 1 - Overview of the work developed

CHAPTER 2: STATE OF THE ART

2.1. BIOPLASTICS

By definition, bio-based polymers are polymers produced from biological renewable resources and polymerized by chemical and/or biological methods. They can be categorized into three main groups: bio-chemosynthetic polymers – like poly(lactic acid) (PLA) and poly(butylene succinate); biosynthetic polymers – PHA being an example; and finally, modified natural polymers – such as starch polymers and cellulose derivatives. For bio-chemosynthetic polymers, the monomers are synthesized biologically and polymerized chemically whereas biosynthetic polymers are completely produced by biological process, from monomers synthesis to polymerization processes (Sudesh 2013). Natural polymers need to be submitted to chemically and/or physically modifications to enhance the polymer structure and improve the thermal and mechanical properties (Hoover et al. 2010).

Still, not all bio-based polymers are biodegradable. Crystalline PLA, cellulose derivatives and polythioesters are some examples of non-biodegradable bio-based polymers. Biodegradable polymers are defined as those that can be degraded hydrolytically and/or enzymatically by microorganisms into simpler substances such as carbon dioxide, water and inorganic compounds (Kumar et al. 2015; Verlinden et al. 2007).

PHA are an excellent candidates to replace conventional plastics not only because of their biodegradable but also due to their wide range of characteristics and properties analogous to petroleum-based plastics, opening doors to various industrial applications (Laycock et al. 2014a).

2.2. POLYHYDROXYALKANOATES

2.2.1. HISTORY

Lemoigne, a french bacteriologist, was the first to report the occurrence of PHA in bacteria in 1926, when he verified the formation of poly(3-hydroxybutyrate) (P(3HB)) in

inclusion bodies of *Bacillus megaterium*. This discovery promoted research about their function as energy reserve and fundamental research of the mechanisms behind PHA biosynthesis began in 1957 with the works of Stanier and Wilkinson (Williamson & Wilkinson 1958; Stanier et al. 1959). For many decades, 3-hydroxybutyrate (3HB) was held as the sole PHA monomeric unit. Only almost 50 years later the presence of other monomer units such as 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) was discovered by Wallen & Rohwedder, 1974. In 1959, W.R. Grace and Company patented a P(3HB) production process using bacteria, making the first attempt at PHA commercialization, but production inefficiencies, poor thermal stabilities and a lack of available extraction technologies limited its application. Nowadays, more than 150 different PHA monomers have been identified (Laycock et al. 2014a).

2.2.2. PROPERTIES

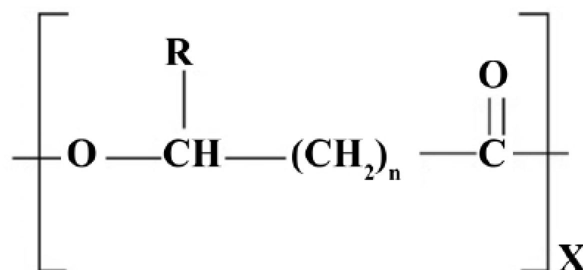
PHA are aliphatic polyesters composed by a large number (600-35000) of hydroxyl fatty acid units, where an ester bond is established between the carboxyl group of one monomer and the hydroxyl group of the other, catalyzed by a PHA synthase (Verlinden et al. 2007; Luengo et al. 2003b). These monomers are usually 3-hydroxyalkanoate (3HAs), which due to the stereo-specificity of the enzymes involved in the biosynthesis are in R configuration (Kumar et al. 2015).

The general structural formula of PHA is displayed in Figure 2, where *X* can reach up to 35000 and the group R varies from C1 (methyl) to C13 (tridecyl). This structure allows countless different variations in length and composition of side chains (Verlinden et al. 2007). The compositional variation can be caused by two factors: endogenous metabolic pathways in the cell and type of carbon substrates supplied to the cultures (Rehm 2003).

Due to their diversity PHA can have a wide range of properties according to monomer composition, microstructure (randomly distributed monomers or organized as block copolymers), and molecular weight distribution (Laycock et al. 2014).

PHA are hydrophobic, water-insoluble, inert and indefinitely stable in air. They are thermoplastic and/or elastomeric, non-toxic, have a semicrystalline structure (degree of crystallinity between 40% and 80%) and high purity within the cell. PHA molecular weights range from $1x10^4$ to $3x10^6$. In comparison with polypropylene, PHA are less resistant to

solvents but more to UV degradation. However, PHA most relevant characteristic is their biodegradability (Laycock et al. 2014).



Number of repeating units, x	Alkyl group, R	Polymer type
1	Hydrogen	Poly(3-hydroxypropionate)
	Methyl	Poly(3-hydroxybutyrate)
	Ethyl	Poly(3-hydroxyvalerate)
	Propyl	Poly(3-hydroxyhexanoate)
	Pentyl	Poly(3-hydroxyoctanoate)
	Nonyl	Poly(3-hydroxydodecanoate)
2	Hydrogen	Poly(4-hydroxybutyrate)
3	Hydrogen	Poly(5-hydroxyvalerate)

FIGURE 2 - General structural formula of PHA (Kumar et al. 2015)

In general, PHA are classified according to the number of carbon atoms present in the monomer in three main groups. Short-chain-length PHA (scl-PHA) contain monomer units with 3 to 5 carbon atoms, medium-chain-length (mcl-PHA) have between 6 and 14 carbons and finally, with more than 14 carbons, long-chain-length PHA (lcl-PHA). Though, some authors may only consider the first two groups of PHA classification, due to the lack of prominence of lcl-PHAs. Variations in the chain length, type and proportions of PHA monomers affect the polymer's thermal properties, like melting and glass transition temperatures, and level of crystallinity (Suriyamongkol et al. 2007; Laycock et al. 2014a; Koller et al. 2013; Reddy et al. 2003).

P(3HB) is the most common PHA. With a melting temperature close to 180 °C and a glass transition temperature around 4 °C, it has good thermoplastic properties. P(3HB) mechanical properties can be comparable to those of polypropylene, however its elongation to break (flexibility) is about 2 – 10% when compared to up to 400% for polypropylene.

Also, its high crystallinity (55 – 80% crystalline) makes it fairly stiff and brittle which can somewhat limit its applications (Lemos et al. 2008).

Copolymers, with incorporation of short-chain monomers other than 3HB, such as 3HV, have a lower polymer crystallinity, due to disturbance in the crystal lattice, and generally also have lower melting and glass transition temperatures when compared to P(3HB). The temperature of PHA degradation is rather insensitive to composition, so lower melting temperatures are advantageous because they allow these copolymers to be processed at lower temperatures than P(3HB). Besides this, these copolymers also have higher melt viscosity and a desirable property for extrusion blowing applications (Reis et al. 2011; Sudesh 2013).

P(3HB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-co-3HV)) mechanical properties can be compared to those of polypropylene and polyethylene, even though they have much lower elongation to break and are more brittle. The physical properties can be manipulated, including for example medium-chain monomers, such as 3-hydroxyhexanoate (3HHx) in PHA copolymers that will make them elastomers. These copolymers will have a much lower melting point and glass transition temperature than the P(3HB) homopolymer and their molecular structure can be, then, compared to soft polypropylene (Reis et al. 2011; Sudesh 2013).

Table 1 shows a comparison between the characteristics of common plastics and PHA polymers.

TABLE 1 – Comparative account on the properties of common plastics and PHA polymers (Kumar et al. 2015; Huang & Tang 2007)

Property	PP	LDPE	PS	PVC	P3HB	P3HB3HV[a]	P3HB3HHx	P3HB3HA[b]
Melting temp. (ºC)	168	123	---*	---*	180	140	127	133
Glass temp. (ºC)	-20	-36	90	110	4	-1	-1	-8
Crystallinity (%)	60	30	---*	---*	60	60	34	45
Young's Modulus (GPa)	1.3	0.4	3.2	3.2	3.5	0.8	0.5	0.2
Tensile Strength (MPa)	36	20	36	46	40	20	21	17
Elongation break (%)	350	530	2	60	5	50	400	680

PP – Polypropylene; LDPE – Low-density polyethylene; PS – Polystyrene; PVC – Polyvinyl chloride
a – P(3HB-co-3HV), mol fraction 80:20; b – P(3HB-co-3HA), mol fraction 94:06; * not available

2.2.3. PRODUCTION MECANISM AND METABOLISM OF PHA

PHA synthesis is a phylogenetically widespread property. More than 300 species of Gram-positive and Gram-negative bacteria and a wide range of *Archea* produce PHA as carbon/energy storage (Laycock et al. 2014).

When there is an excess of carbon and the growth is limited externally - by absence of other essential nutrients (such as oxygen, phosphorous or nitrogen) - or internally – by lack of anabolic enzyme levels or activity, bacteria synthesize PHA intracellularly as insoluble cytoplasmic inclusions (Anderson et al. 1990). As PHA are accumulated in inclusion bodies, the osmotic state of the cell does not change and therefore PHA can be stored at high concentrations within cytoplasm (8–13 granules per cell, with diameters from 0.2 to 0.7 μm) (Laycock et al. 2014; Sudesh et al. 2000), as shown in Figure 3.

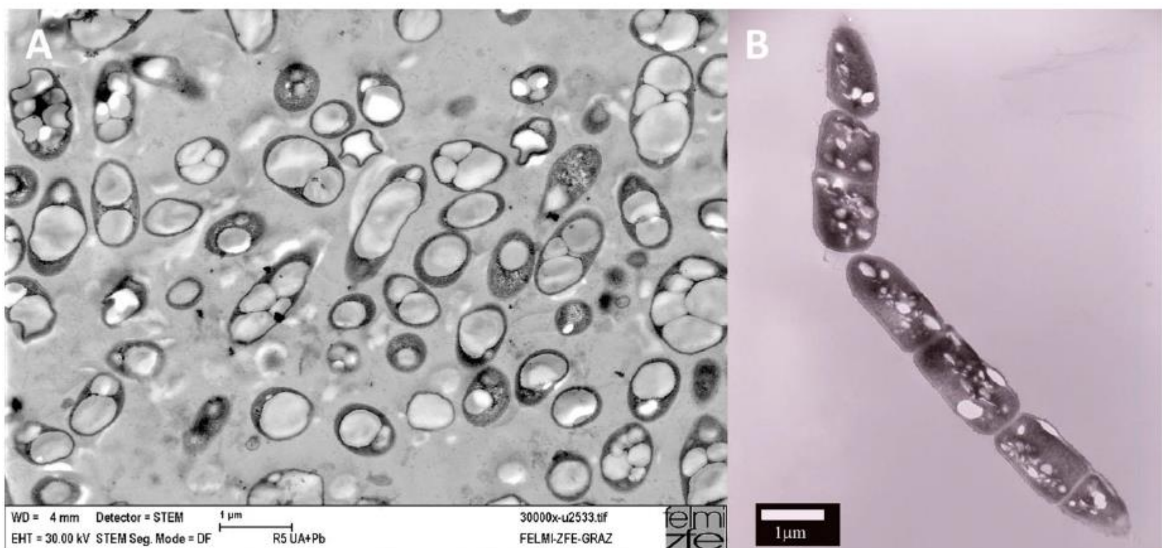


FIGURE 3 – Scanning transmission electron microscopy (STEM) pictures of cell with PHA inclusion bodies (Koller & Rodríguez-Contreras 2015).

Most pure culture PHA-producing bacteria use the Entner-Doudoroff pathway for carbohydrate catabolic degradation originating pyruvate, energy - adenosine triphosphate (ATP) - and reducing equivalents - reduced nicotinamide adenine dinucleotide (NADH). When there is nothing limiting the growth, pyruvate is converted to acetyl-CoA. The compound enters the tricarboxylic acid (TCA) cycle to be oxidized into CO_2 with simultaneous formation of anabolic precursors, additional energy, and reducing equivalents.

Under growth-limiting conditions, acetyl-CoA can be converted into P(3HB), by inhibition of the TCA cycle enzymes, for instance (Dawes & Senior 1973).

There are three main enzymes involved in the biosynthesis of P(3HB) from acetyl-CoA: 3-ketothiolase (PhaA) catalyzes the condensation of two units of acetyl-CoA into acetoacetyl-CoA; acetoacetyl-CoA reductase (PhaB) promotes the reduction of acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA; which is finally incorporated by PHA synthase (PhaC) into the polymer chain as (3HB) (Luengo et al. 2003).

Short-chain organic acids (SCOA) can also be precursors for PHA biosynthesis by their activation to the corresponding acyl-CoA molecule, which will originate different HA monomers, as shown in Figure 4, section (c). If acetic acid is used as carbon source it will originate two acetyl-CoA units that, as described above, can generate HB monomers (Lemos et al. 2008).

Propionate is the precursor of either propionyl-CoA or to acetyl-Co-A (by decarboxylation of propionyl-CoA). The first can, when combined with one acetyl-CoA, generate hydroxyvaleryl-CoA and lead to HV formation. Acetyl-CoA synthesis, compulsory for HV production, can occur via five different metabolic pathways: the methylmalonyl-CoA pathway, the α-hydroxyglutarate pathway, the citramalate pathway, the acryloyl-CoA pathway and the 2-methylcitric acid pathway (Horswill and Escalante-Semerena, 1999; Lemos et al., 2003). Even though the main pathway for propionate degradation is HV production other monomers can also be synthesized, namely: HB units - from the condensation of two acetyl-CoA units into hydroxybutyryl-CoA, 3-hydroxy-2-methylvalerate (3H2MV) – from two propionyl-CoAs and 3-hydroxy-2-methylbutyrate (3H2MB) – by combining one propionyl-CoA with one acetyl-CoA (Luisa S Serafim et al. 2008).

Butyrate and valerate can also be used as substrates for PHA synthesis, since butyrate has the four-carbon backbone of HB and valerate the five-carbon backbone of HV. Butyrate would originate butyryl-CoA that could subsequently be converted into hydroxybutyryl-CoA for HB production. Valerate would be converted into valeryl-CoA, then into hydroxyvaleryl-CoA which will lead to HV production (Lemos et al. 2006).

PHA can also be synthetized by three other metabolic pathways: ß-oxidation of medium and long-chain length fatty acids; *de novo* fatty acid synthesis can originate intermediates for PHA production; oxidation of alkanes will lead to the corresponding

alkanoate formation which can be activated to the corresponding acyl-CoA and used to biosynthesize PHA, described in Figure 4, sections (d), (b) and (e), respectively (Serafim et al. 2008; Reis et al. 2011). Although those are the main metabolic pathways, many others can lead to the microbial production of scl-PHA and mcl-PHA, as reviewed by Tan et al. (2014).

In general, metabolic pathways for PHA synthesis by MMC are assumed to be similar to those described for pure cultures. It is also expectable that MMC are capable of producing a wider range of PHAs types since they have diverse organisms which are likely to employ more pathways for PHA biosynthesis.

Figure 4 represents a scheme of the referred metabolic pathways.

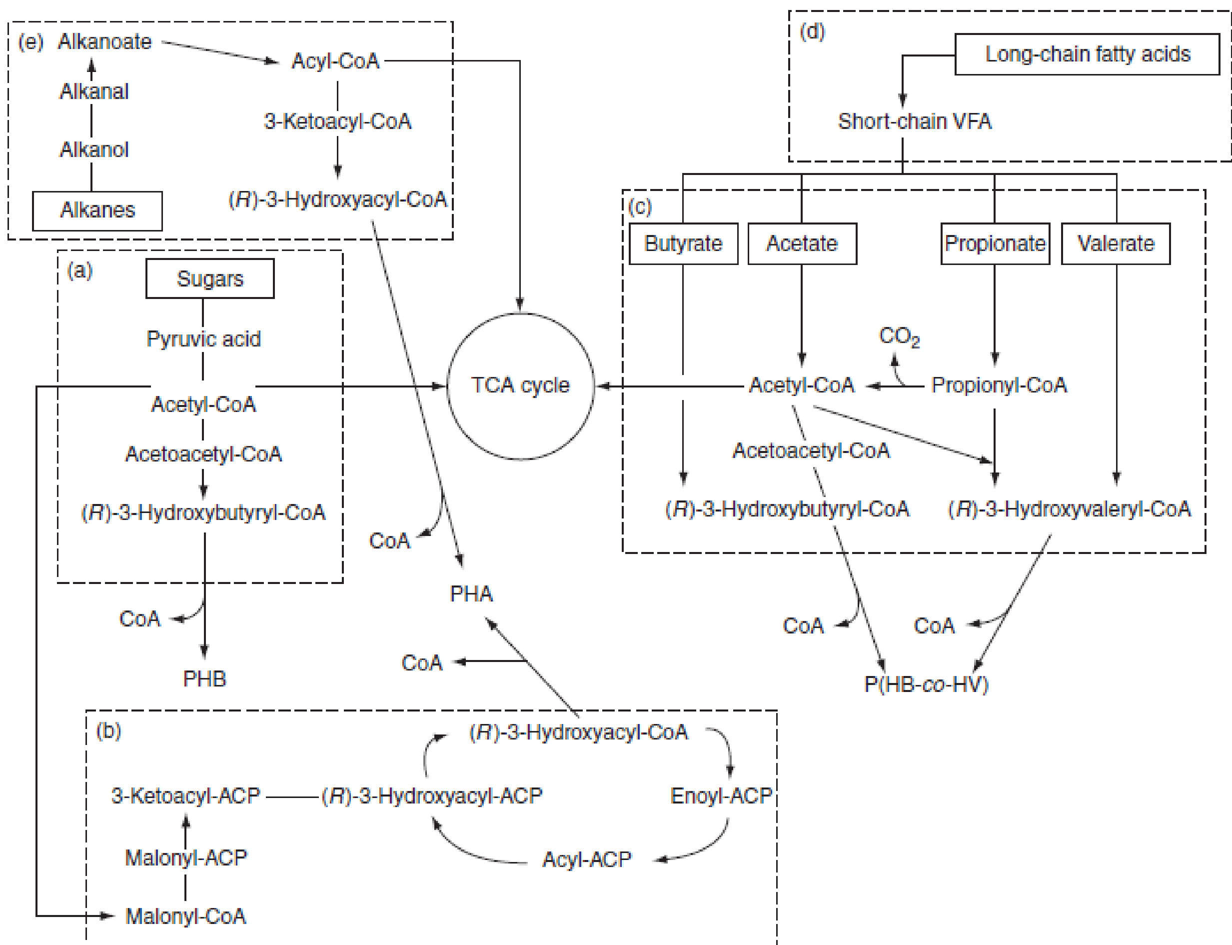


FIGURE 4 - Metabolic pathways involved in PHA synthesis (Reis et al. 2011).

2.2.4. BIODEGRADABILITY

As said before, one of the most interesting characteristics of PHAs is their biodegradability (Sudesh & Iwata 2008).

During the degradation process, normally PHA suffers hydrolysis of ester bonds, catalyzed by intracellular or extracelluar PHA depolymerase enzymes secreted by various bacteria and fungi in order to break down the biopolymer into low molecular weight products that can be assimilated and metabolized by the microorganisms. Under aerobic conditions the final products of degradation of PHA are carbon dioxide and water while in anaerobic conditions methane is produced (Kumar et al. 2015; Guérin et al. 2010). Several studies reported the biodegradation of pure culture PHA in different environments – aquatic, activated sludge, compost and soil (Weng et al. 2011; Briese et al. 1994; Volova et al. 2010).

Arcos-Hernandez et al. (2012) conducted a study on the biodegradability of PHA produced by MMC in a soil based environment. 90% of biodegradation was achieved at between 10.7 and 22.2 months. Figure 5 shows the physical apparent degradation after 15.9 weeks (Arcos-Hernandez et al. 2012).

FIGURE 5 - Physical degradation of PHA films from mixed culture after 15.9 weeks of incubation (Arcos-Hernandez et al. 2012).

The biodegradation process is dependent on both environmental factors - temperature, moisture, oxygen, pH – and polymer characteristics – structure, crystallinity, molecular weight, and, in the case of copolymers, the monomeric composition (Sudesh et al. 2000; Verlinden et al. 2007; Arcos-Hernandez et al. 2012).

P(3HB) can be hydrolytically degraded to a normal constituent of human blood and, because of its high crystallinity and absence of PHA depolymerase in humans, it degradates at a very slow rate. This makes P(3HB) a potential material for biomedical applications (Nair & Laurencin 2007). On the other hand, P(3HB-*co*-3HV) has lower crystallinity and higher

rate of degradation which suggests it as a better temporary substrate for tissue engineering such as bone tissue and epithelial tissue (Chen & Wu 2005; Torun Köse et al. 2003).

2.2.5. CURRENT INDUSTRIAL PRODUCTION

Many organisms - like bacteria, fungi and plants - were already the focus of PHA production studies (Chen 2009). Most of the industrial processes for PHA production implemented so far use pure microbial cultures, either wild or genetically modified strains – *Cupriavidus necator, Alcaligenes latus, Aeromonas hydrophila, Bacillus spp., Pseudomonas putida, Pseudomonas oleovorans* and genetically modified *Escherichia coli* are some examples. The great advances in genetic engineering in recent years allowed the construction of recombinant strains with desirable characteristics, namely fast growth and high accumulation rates (that can currently go up to 90% of the cell dry weight), ability to use inexpensive substrates and simple polymer purification (Reis et al. 2003; Salehizadeh & Van Loosdrecht 2004; Dias et al. 2006; Akaraonye et al. 2010).

Almost all established industrial processes use pure sugars or sugar-based compounds as substrate like corn sugar, sugar beet and sugarcane, which have a high market price. The cost of the carbon source represents around 30-50% of the total PHA production price (Kumar et al. 2015).

The two aspects mentioned above are related to two major cost drivers of PHA production – energy for sterilization and substrate cost. Another very important cost driver is downstream processing since a substantial number of unitary operations are necessary and can implicate a considerate amount of chemicals and energy demand (Serafim et al. 2008).

Nonetheless many advances were made to significantly reduce process costs. In recent years, technological advances, strain development, exploitation of cheap substrates and novel operational strategies led to lower prices. In 2006, the market price of PHB was around 10-12€ per kilogram and by 2014 its price was reduced to 1.5-5€ per kilogram. Other copolymers prices, like P(3HB-co-3HV) and P(3HB-co-3HHx), range from 3-14€/kg. Although current prices are not low enough to be directly competitive with petroleum-based plastics they consolidate PHA's market position (Kosior et al. 2006; Chanprateep 2010; Koutinas et al. 2014).

Several companies are already commercializing PHA from which can be highlighted: Metabolix Inc. (USA), Shenzhen Ecomann Technology Co. Ltd. (China), Tianjin GreenBio Materials Co. Ltd. (China), Meredian Inc. (USA) and Biomer (Germany) (Kumar et al. 2015). Table 2 summarizes some examples of PHA large scale production in the last years.

2.2.6. APPLICATIONS

When it comes to applications, PHAs raised the attention of different industrial branches. This family of versatile biopolymers is suitable for many applications and markets. PHAs uses are not limited to those of conventional plastics. Their biocompatibility and biodegradability allows them to be consider for biomedical uses (Koller et al. 2014). Some of the most relevant applications of PHA are described below.

2.2.6.1. PACKAGING AND COMMODITY ITEMS

The most known and least pretentious application of PHA is in replacement of conventional plastics. One of the areas where compostable plastics are wanted is packaging. Especially when it is used for a short period, like in food packaging and waste bags, and disposed creating enormous piles of waste. For food packaging, PHA were shown to be at least as good as high-density polyethylene, the classical package material. PHA can also be used in the production of various daily commodities like razors, diapers, hygiene products, or cups and dishes. In agriculture, mulch films can be produced from PHA. This biopolymers can also be used for more durable applications such as cars and electronic components (Sudesh & Iwata 2008; Haugaard et al. 2001; Chen 2009).

2.2.6.2. BIOMEDICINE

PHA biocompatibility makes them interesting candidates for applications in the medical field, a fact that is sustained by the continuous and growing research in biomedicine using this polymer. PHA were successfully investigated for *in vivo* applications, bone implant materials, for tissue engineering, as implants, surgical pins, screws, meshes and sutures and as carrier matrices for controlled drug release. Their use was also reported in the

TABLE 2 – Examples of PHA large scale production (Koller et al. 2014)

Company	Country	Production (since/until)	Microbial production strain	Substrate(s)	Type of PHA	Trade mark	Annual production scale
Chemie Linz	Austria	Late 1980s to early 1990s	*Alcaligenes latus* DSM1124 (today: *Azohydromonas lata*)	Glucose from carbohydrate feedstocks	PHB	–	< 50 t
Biomer	Germany	1993	*Cupriavidus necator*	Glucose from corn starch	PHB	Biomer™	500 t–1000 t
Imperial Chemical Industries (ICI) (Later: Zeneca, Monsanto)	UK	1976–1998	*Alcaligenes eutrophus* (today *Cupriavidus necator*)	Glucose from carbohydrate feedstocks	PHB, later also PHBHV	BIOPOL®	800 t (later phase under Monsanto)
Metabolix (with Monsanto technology)	USA	1980-ongoing	n.r.	-	PHBHV and others	-	n.r.
Telles (joint venture of Metabolix and ADM)	USA	2007–2012	n.r.	-	PHBHV and others	Mirel™ and Mvera™	planned 50 000 t
Bio-On	Italy	-	n.r.	Sugar co-products or sugar waste material (molasses)	n.r.	MINERVPHA™	10 000 t
Tepha Inc.	USA	2007-ongoing	n.r.	-	P4HB, P(3HB-co-4HB), *mcl*-PHA	TephaFLEX™	n.r.
Polyferm Canada	Canada	ongoing	wild type bacteria	Vegetable oils, sugars	*mcl*-PHA	VersaMer™	-
Tianjin Green Bioscience & DSM	PR China	2004-ongoing	n.r.	-	P(3HB-co-4HB)	GreenBio™	10 000 t
Tianan	PR China		*Cupriavidus necator*	-	PHBHV	Enmat™	100 t–1000 t
PHB Industrial/ Copersucar (PHBISA)	Brazil	1995-ongoing	*Alcaligenes eutrophus* (today *Cupriavidus necator*); *Burkholderia sacchari*	Cane sugar	PHB and PHBHV	BIOCYCLE™	100 t (capacity 5000 t)

Note: n.r. not reported (information not provided by manufacture

production of highly sophisticated surgical articles like artificial blood vessels and vein valves, spinal fusion cages, bone marrow scaffolds, joints, and meniscus regeneration devices (Chen & Wu 2005; Zinn et al. 2001; Valappil et al. 2006).

However for this type of applications, high purity is a pre-requisite in order to prevent negative impacts on the human organism. Considering that during microbial production the occurrence of unwanted byproducts - like endotoxins - could happen, downstream processing must assure a sufficient removal of impurities (Valappil et al. 2006).

2.2.6.3. OTHER INOVATIVE APPLICATIONS

Hydrolysis of PHA will lead to the production of chiral, optically pure *R*(-)-configured bifunctional monomers that can be used as building blocks for syntheses of fine chemicals and marketable products such as pheromones, aromatics, vitamins or antibiotics, or can even be used as pharmaceutically active compounds. Furthermore, they can also be used as 'functional materials' for different niche applications, like heat sensitive adhesives, latex materials, or smart gels. PHA can act as carrier materials and degradable matrices for release of active agents - including drugs, hormones, pesticides, antibiotics, dyestuffs, or flavors - at controlled rates (Sudesh & Iwata 2008; Chen 2009; Ren et al. 2005).

Table 3 provides a resume of the some of the application fields were PHA can be involved.

2.3. MIXED MICROBIAL CULTURES

Mixed microbial cultures (MMC) are populations that operate in open biological systems. Their composition varies according to the substrate and operational conditions imposed on the bioreactor.

Activated sludge systems used in wastewater treatment plants (WWTPs) are some of the most well-known MMC and the first where PHA accumulation was reported. In 1974, Wallen and Rohwedder observed PHA heteropolymers in the chloroform extracts of activated sludge from an enhanced biological phosphorus removal (EBPR) system. Since then, many studies proved MMC capacity for PHA storage (Wallen & Rohwedder 1974; Van Loosdrecht et al. 1997; Valentino et al. 2015)

TABLE 3 - Applications of PHA

Industry	Applications	Reference
Packaging materials	Containers for commodity products, packaging films, disposable products, medical surgical garments, textiles, compostable bags and lids and tubs for thermoformed articles	(Vincenzini & De Philippis 1999; Chen 2010)
Agriculture field	Controlled release of fertilizers, plant growth regulators, pesticides and herbicides, seed encapsulation and covering foils	(Vincenzini & De Philippis 1999)
Medical field	Devices including sutures, surgical mesh, surgical pins, repair patches, cardiovascular patches, slings, atrial septal defect repair devices, guided tissue repair and regeneration devices, bone marrow scaffolds, tendon repair devices, ocular cell implants, skin substitutes, articular cartilage repair devices, ligament and tendon and bone graft substitutes, vein valves , hemostats and wound dressings, etc.	(Vincenzini & De Philippis 1999; Saharan & Sharma 2012; Chen 2010)
Pharmaceutical field	Drug delivery systems, retarded drug release	(Vincenzini & De Philippis 1999; Hazer et al. 2012)
Biofuels	3-hydroxybutyrate methyl ester and MCL 3-hydroxyalkanoate methyl ester resulted fromesterification of PHB and MCL-PHA could be used as biofuels	(Chen 2010)
Chiral chromatography	Stationary phase for columns	(Vincenzini & De Philippis 1999)
Miscellaneous	Autoseparative air filters, fiber-reinforced	(Vincenzini & De Philippis 1999)

Another interesting characteristic of MMC, especially those from WWTPs, is their ability to quickly adapt to different conditions since they experience frequent changes in substrate composition. These microorganisms must be able to store the carbon source in a short period of time in order to consume it later, during periods of absent of external carbon source (Serafim et al. 2008).

PHA production by MMC is an alternative to reduce the high production costs associated to pure cultures' technology. With MMC no sterilization is necessary (allowing energy saving), fermentation equipment is less expensive since there is less need for control

and the culture is able to adapt to various complex waste feedstocks, which may lead to an operation cost reduction up to 50%. Since there is less need for process control the cost of the monitorization equipment is also inferior (Reis et al. 2011; Serafim et al. 2008).

Nonetheless, MMC still have some drawbacks compared to pure cultures. Volumetric productivities are usually lower compared to pure cultures due to low cellular concentrations. And while in pure cultures the maximum production can go over 90% of the cell dry weight (cdw) with MMC these values are usually lower. However recent studies have already achieved accumulation rates comparable to those of pure cultures. Johnson et al. (2009) observed a P(3HB) production of 89 % cdw in less than 8 h using acetate as substrate; Jiang et al. (2009) obtained 77 % cdw of PHA using fermented paper mill wastewater and 74.6 % cdw of PHA content was reported by Albuquerque et al. (2010) with fermented molasses as substrate. These results show that through process optimization and technological advance PHA production using MMC can become a reality in near future, and one of much interest due to its simplified fermentation conditions and environmental advantages (Chen et al. 2015).

2.4. PROCESS OPERATION: THREE-STAGE PROCESS

An effective PHA accumulation process by MMC relies on efficient culture selection and high amounts of accumulated biopolymer and should be based on adequate reactor operational strategies. Physical separation of the steps involved in this process is crucial to impose different optimal conditions required for each process instead of compromising conditions. For this reason most of the processes for PHA production using MMC undergo a three-stage process, comprising: (1) acidogenic fermentation of the carbon source to produce volatile fatty acids (VFA) mixtures that will serve as precursors of PHA biosynthesis; (2) culture selection, by imposing high selective pressure for microorganisms with PHA storage ability and finally (3) PHA production stage were the selected microorganisms accumulate PHA at maximum capacity, as shown in Figure 6 (Duque et al. 2014; Chen et al. 2015).

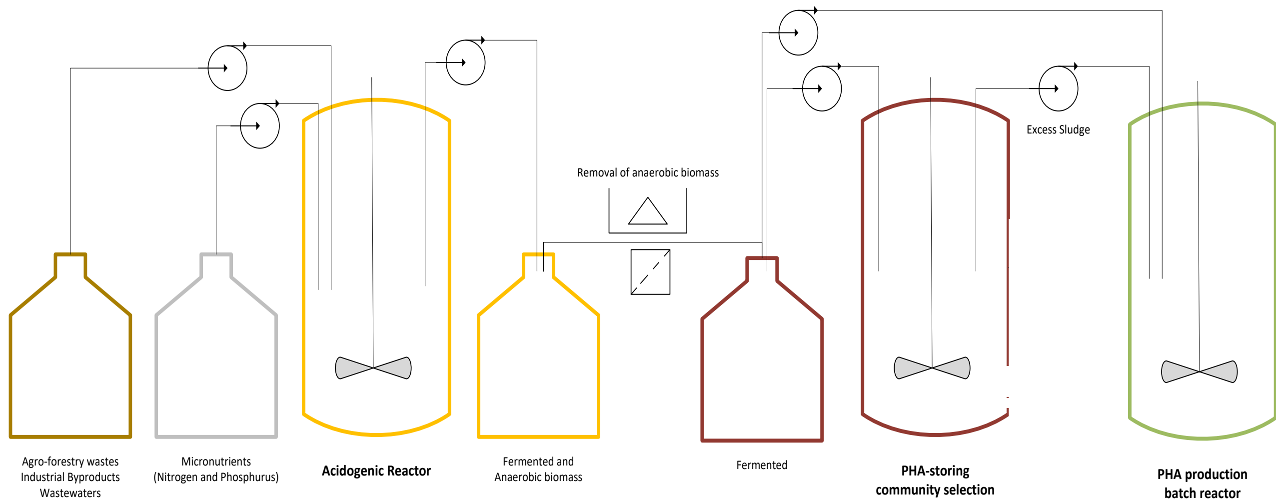


FIGURE 6 – Example of three-stage process, adapted from (Queirós et al. 2015).

2.4.1. ACIDOGENIC FERMENTATION

Anaerobic digestion (AnD) is a processes where complex organic compounds are fermented into intermediate products that are lately converted into methane and carbon dioxide, by a series of sequential biochemical processes. The process is composed of four stages – hydrolysis, acidogenic fermentation, acetogenesis and methanogenesis – executed in that sequence by distinct groups of organisms. In hydrolysis complex carbon sources, like polysaccharides, lipids and proteins, are fragmented into simpler monomers, by acidogenic bacteria. Those same bacteria promote the fermentation of the monomers into SCOA, ethanol, carbon dioxide and hydrogen, in acidogenic fermentation. In acetogenesis, SCOA are converted into acetate, hydrogen and carbon dioxide, by acetogenic bacteria. And finally, methanogenic organisms convert the products of acetogenesis into carbon dioxide and methane (Demirel & Yenigün 2002; Singhania et al. 2013).

Most agro-industrial feedstocks are rich in carbohydrates and other compounds that cannot be directly converted into PHA or that are preferably transformed into glycogen which makes them less suitable substrates for PHA production by mixed cultures (Carta et al. 2001; Dircks et al. 2001; Lemos et al. 2006; Queirós et al. 2015). This setback can be

overcome by fermenting the residues into SCOA, in an acidogenic fermentation process, for this to happen the other steps of AnD must be inhibited. Generally, acidogenic fermentation is operated under continuous mode so that the culture can become acclimatized to the feedstock. However, to minimize the risks of shock loading and washout problems, biofilm systems have been presented as an alternative to suspended growth reactors (Morgan-Sagastume et al. 2014; Bengtsson et al. 2008a).

As showed before, SCOA can be metabolically converted to PHA by MMC and many studies on these biopolymers production were carried out using organic acids as feedstock (Lemos et al. 2006; Lee et al. 2014). From a metabolic perspective SCOA are more energetically advantageous substrates than sugars, since β-oxidation generates more equivalent chemical energy than the oxidation of a molar equivalent of glucose (Laycock et al. 2014).

Since monomer composition of PHA is related to their mechanical and thermal properties and can consequentially determine the type of application, variations in the type and proportions of SCOA fed to the culture can lead to a great variety of monomer compositions and influence the final product, which is another interesting advantage of using SCOA. By manipulating the acidification process in order to obtain different SCOA profiles, residues can be transformed into tailored made biopolymers (Lemos et al. 2006; Cerrone et al. 2014).

Some studies, using enriched MMC, have already showed how different synthetic SCOA profiles and accumulation conditions can influence the type of PHA monomers produced. Dionisi et al. (2004) reported that when propionate was used as a sole carbon source P(3HV) was produced, whereas the same culture produced P(3HB) from acetate (Dionisi et al. 2004). Lemos et al. (2006) studied the independent use of acetic, propionic, butyric and valeric acids, obtaining different polymer compositions, as shown in Table 4. As well as acetate and propionate mixtures enriched in different organic acids (Lemos et al. 2006). The influence of different organic acids ratios was studied by Hu et al. (2005). In this work, increasing the ratio of propionate in a mixture of acetate and propionate fed to activated sludge led to the increase of the (3HV) fraction in the produced P(HB-co-HV) copolymer (Hu et al. 2005).

The outcomes of some studies in the influence of SCOA in the type of monomer produced by enriched MMC are summarized in Table 4.

TABLE 4 - Influence of SCOA in the type of monomer produced by enriched MMC

SCOA fed	Polymer produced	Selection Process	Ref.
Acetate	PHB	ADF	(Serafim et al. 2004; Fradinho et al. 2014; Dionisi et al. 2004)
Acetate	PHV	ADF	(Lemos et al. 2008)
Propionate	PHV	ADF	(Dionisi et al. 2004)
Propionate	P(HB-co-HV)	ADF	(Fradinho et al. 2014; Lemos et al. 2006)
Butyrate	PHB	ADF	(Fradinho et al. 2014; Lemos et al. 2006)
Valerate	P(HB-co-HV-co-HMV)	ADF	(Lemos et al. 2006)
Acetate Propionate	P(HB-co-HV)	AN/AE	(Hu et al. 2005)
Acetate Propionate Lactate	P(HB-co-HV)	ADF	(Dionisi et al. 2004)
Acetate Propionate Butyrate Valerate	P(HB-co-HV)	ADF	(Duque et al. 2014; Albuquerque et al. 2012)

Many types of complex substrates were already submitted to fermentation to be used as substrate for PHA, like sugarcane molasses, paper mill wastewater and cheese whey (Bengtsson et al. 2008; Carvalho et al. 2014).

However studies showing how the manipulation of acidogenic conditions can lead to different monomer composition are still scarce. Bengtsson et al. (2008) showed that increasing the hydraulic retention time (HRT) in the acidification step led to an increase of propionate in the VFA profile, and consequently improved the HV content in the copolymer P(HB-co-HV). The influence of pH in the composition of SCOA produced from wastes was studied by both Bengsston et al. (2008) and Albuquerque et al. (2007), their works showed that by increasing the pH they could promote the production of certain SCOA, which changed according to the substrate used in the study, and verified the consequent changes in monomer composition (Bengtsson et al. 2008; Albuquerque et al. 2007).

2.4.2. CULTURE SELECTION

The main goal of the selection step is to guarantee good PHA accumulation performance, during the last step, by obtaining from the initial activated sludge a culture enriched in microorganisms that have high PHA storage capacity. This strategy will eliminate organisms with low or no storage ability that would have a negative impact in the process productivity and on downstream processing, increasing extraction costs (Queirós, Lemos, et al. 2015; Marang et al. 2014).

However, cultures with high storage capacity may be unstable and sensitive to variations in operating parameters and feedstock composition (Queirós 2015). Therefore, the selection step should aim to achieve a compromise between maximizing the cell PHA content and assure the culture's stability (Serafim et al. 2008). A selection process that results in a culture with a diverse population of biopolymer accumulating organisms is more robust and adaptable, and consequently, more stable than a monoculture (Reis et al. 2011).

Majone and colleagues (2006) showed the importance of the selection step by comparing the storage capacity of an activated sludge with that of an enriched MMC. The enriched culture had a PHA storage rate almost 20 times superior compared to activated sludge and PHA content was 4 times higher. In current processes the enriched culture is composed by 80-90% of organisms with PHA storage capacity, with high PHA content and storage productivity (Majone et al. 2006; Albuquerque et al. 2010; Jonhsson et al. 2009).

Another important thing to consider in this step is biomass volumetric productivity, since selection conditions tend to lead to low cell densities by limitation of the cultures' primary metabolism. Subsequently, volumetric productivity will be limited on the PHA accumulation stage. There is continuous development of culture selection and reactor operation techniques but it is still a challenge to develop a strategy to select for a culture with both high growth rate - operating at high organic loading rate (OLR) and short retention time (SRT) - and high storage – efficient selective pressure (Reis et al. 2011).

2.4.2.1 SELECTION STRATEGIES

2.4.2.1.1. AN/AE PROCESS

In EBPR systems were the first systems where PHA storage was observed. These systems are operated under alternating anaerobia/aerobic cycles and polyphosphate-accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) are the two main groups of organisms selected. When submitted to anaerobic conditions these cultures convert the carbon source into PHA with simultaneous consumption of glycogen, another storage polymer. Under aerobic conditions, and when there is no more substrate available, PHA previously stored is used to growth, maintenance and glycogen biosynthesis (Serafim et al. 2008). GAOs are more suited for processes where the main goal is PHA accumulation since they have a higher robustness (Dai et al. 2007).

Several studies already used this selection process with real complex substrates. Coats et al. (2007) used fermented wastewater in an AN/AE system for the selection of cultures with PHA storage ability, having achieved 53% cdw of PHA content (Coats et al. 2007). Pisco et al. (2009) used fermented sugar cane molasses for enrichment of a GAO culture that showed ability to accumulate PHA up to 37.3 % cdw in an accumulation test (Pisco et al. 2009). One of the best results was reported by Jiang and colleagues (2009), a 72.9% PHA content was achieved with waste-activated sludge alkaline fermentation liquid as the carbon source (Jiang et al. 2009).

2.4.2.2.2. AEROBIC DYNAMIC FEEDING

Significant PHA accumulation was observed in activated sludge used for aerobic wastewater treatment working in process configurations where there was periods of excess and lack of carbon source. Submitting MMC to consecutive periods of external substrate accessibility (feast) followed by periods of unavailability (famine) was proved to enhance the PHA storage capacity. This process, currently designated aerobic dynamic feeding (ADF), or “feast and famine” (FF), creates dynamic conditions and it is the most common

process used for culture enrichment in PHA-storing microorganisms (Albuquerque et al. 2011; Dionisi et al. 2005; Serafim et al. 2004).

During the period of excess of external substrate, the carbon uptake could be directed to cell growth and PHA storage and in the next period, after substrate exhaustion, the stored polymer is used as carbon and energy sources. This privileges microorganisms that are quicker to store PHA. Usually the storage phenomenon is dominant (≥ 70% on a C-mol basis) when compared to growth (Beun et al. 2002; Serafim et al. 2004). During the famine period, there is a decrease of the amount of intracellular components necessary for cell growth, like RNA and enzymes. When substrate is available again, these components may not be in the necessary concentration to ensure maximum growth rates. On the other hand, PHA storage requires fewer enzymes allowing higher production rates and permitting a faster way of consuming the available substrate during the feast phase (Dionisi et al. 2006b).

In recent years, many studies showed that ADF can successfully be used to select cultures with good accumulation capacity using a wide range of complex carbon sources. Moita and Lemos (2012) used bio-oil resulting from the fast-pyrolysis of chicken beds as substrate and the maximum PHA content obtained was 9.2% cdw (Moita & Lemos 2012). Hardwood spent sulfite liquor (HSSL) was also used by Queirós et al. and an accumulation of 67.7% was achieved during the batch accumulation tests (Queirós et al. 2014). Morgan-Sagastume et al. 2015 tested a pilot-scale process using municipal wastewater under FF selection to select a PHA accumulating culture while treating readily biodegradable chemical oxygen demand (COD) from influent water, accumulation rates were up to 50% of cdw (Morgan-Sagastume et al. 2015).

The two main differences between AN/AE and FF are related to cell growth rate and polymer composition. In AN/AE biomass grows only from the accumulated polymer whereas in FF cell growth is based upon both external substrate, in feast phase and PHA storage, in famine phase. For this reason AN/AE has lower productivities than FF. On the other hand, glycogen can be degraded into precursors for PHA synthesis, which could mean that AN/AE processes could produce a wider range of polymer composition (Serafim et al. 2008; Bengtsson et al. 2008a).

2.4.2.3. OPERATIONAL PARAMETERS

The majority of existing studies on PHA accumulation using MMC, and operating under an ADF strategy, use sequenced batch reactor (SBR) as reactor configuration. The SBR is a compact system and can be operated in consecutive cycles of feed, reaction phase (culture selection), settling and discharge.

Several factors could influence the selective pressure of PHA accumulating organisms in the reaction phase. Main factors include type of substrate, SRT, hydraulic retention time (HRT), feast and famine ratio (F/F ratio), cycle length, pH and temperature.

2.4.2.3.1. SLUDGE RETENTION TIME

The operation parameter than can be more easily related to the culture's specific growth rate is SRT. By definition, SRT indicates the mean residence time of microorganism in the reactor. This means that only organisms that are able to reproduce themselves during this time can be enriched in the system. High SRTs allow the enrichment of slowly growing bacteria, consequently a lower fraction of the substrate will be used for growth, and therefore a higher storage yield (Beun et al. 2002).

According to the works of Beun et al. (2002), the yield of PHB from acetate under excess nutrients was constant and independent of the specific growth rate for SRTs higher than 2 days. Bellow this time they verified a decrease in PHB storage yield and productivity. However some studies successfully selected cultures with good storage capacity using low SRT (1 day) (Beun et al. 2002; Lemos et al. 2006; Dionisi et al. 2006b).

Nonetheless it is important to balance the need for both selective pressure for PHA storage and high growth when selecting the operational SRT (Reis et al. 2011).

2.4.2.3.2. FEAST AND FAMINE RATIO

F/F ratio reflects the relation between the duration of the feast and the famine phase. This ratio can be manipulated through changes in the OLR or influent substrate concentration. Famine phase must be long enough to ensure the internal growth limitation necessary to induce PHA production, otherwise the culture will be better fit to grow instead of accumulate polymer during the feast phase. For this reason, low F/F ratios will lead to a

better physiological adaptation of the culture favoring the accumulation during the feast period. Usually low F/F are obtained by lowering OLR in order to have selective pressure for microorganisms that have PHA storage capacity (Beun et al. 2002; Serafim et al. 2004).

The influence of several F/F ratios (0.10-1.15) on an SBR performance fed with a mixture of synthetic organic acids was studied by Dionisi and colleagues (2006). They verified a direct effect in both PHA storage and growth. For low F/F ratios (up to 0.26) they observed high storage response, whilst for F/F ratios higher than 0.90 growth response was more significant. For intermediate values there was instability (Dionisi et al. 2006a). Generally the culture PHA accumulation capacities decreased with the increase in F/F ratio (Reis et al. 2011). Changes in F/F ratio were achieved by manipulating OLR. Albuquerque et al. (2010) did a similar study but in different conditions - variations in influent substrate concentration were used to alter the F/F ratio and SRT was ten times superior - and a similar conclusion was reached (Albuquerque et al. 2010).

2.4.2.3.3. SUBSTRATE CONCENTRATION

Substrate concentration in the influent influences the kinetic of substrate consumption and consequentially PHA storage. Increasing the substrate concentration will lead to higher substrate uptake rates until a maximum is reached - cellular maximum specific substrate consumption capacity. High substrate concentrations not only not contribute to an increase of substrate consumption but may also cause inhibition of the culture as seen in many studies (Serafim et al. 2004; Takabatake et al. 2000).

Low substrate concentrations are also of no advantage since substrate uptake will be limited by substrate concentration. In this case PHA producing organisms' competitive advantage of rapid substrate intake would be lost and the selection process will be less efficient (Albuquerque et al. 2010; Dionisi et al. 2006b; Bengtsson et al. 2008b).

2.4.3.4. pH AND TEMPERATURE

PHA-storing microrganisms selection and accumulation are also affected by pH and temperature. However, the influence of these parameters on the MMC culture selection stage is not very clear since they are highly dependent on the microbial community. In spite of the

effect of temperature in PHA production studies on its effect on the selection of MMC are scarce and do not lead to according conclusions (Krishna and Van Loosdrecht 1999; Johnson et al. 2010; Jiang et al. 2010).

Most of the selection processes occur at pH values equal or slightly higher than 7. Regarding the influence of pH on culture selection we can assume that significant variations of pH will lead to the selection of different microbial communities. Chua et al. (2003) reported that the storage capacity of a microbial culture selected at pH 7 and 8 was the same. Contrastingly, studies conducted by Villano and colleagues (2010) showed that storage rates and yields of cultures selected at different pH decreased as pH increased from 7.5 to 9.5 (Villano et al. 2010; Chua et al. 2003).

2.5.3. ACCUMULATION STEP

In the accumulation step the selected MMC is submitted to batch experiments that increase PHA accumulation, using the same substrate as the one used in the selection phase. The separation of these processes allows the operation under optimal conditions.

2.5.3.1. OPERATIONAL PARAMETERS

PHA storage yield is influenced by many parameters, from which we can highlight: nitrogen and phosphorus concentrations, oxygen concentration, pH and temperature (Dias et al. 2006).

2.5.3.1.1. NITROGEN AND PHOSPHORUS CONCENTRATIONS

The influence of manipulation of nutrient levels, particularly N and P, in order to improve MMCs volumetric PHA productivity was not systematically considered in the research literature. The existing studies on the effects of N and P on the performance of MMC PHA accumulations may be roughly categorized into those where biomass selection

occurred at shorter (1 day) and longer (7-10 days) SRTs (Chinwetkitvanich et al. 2004; Wen et al. 2010; Valentino et al. 2015)

For shorter SRT, PHA accumulations were shown to occur within a range of N levels. Some studies reported improvement of specific storage rates and productivities with the increase of N levels but the maximum PHA content was achieved under N starvation. Dionisi et al (2005, 2004) reported PHA productivities to be the highest in case of N-starvation (61% gPHA/g volatile suspended solids (VSS)) than with N-excess (N/COD> 33 mg/g; 50% g PHA/g VSS). Johnson et al. (2010) also obtained the maximum biomass PHA content, 89% g PHA/g VSS, under N-starvation, but in contrast also achieved the highest PHA productivities (0.15 g PHA/L.h) in these conditions (Dionisi et al. 2005; Dionisi et al. 2004; Johnson et al. 2010).

In case of longer SRTs restricting N and P levels has led to more favorable PHA storage rates and yields, biomass PHA contents, and PHA productivities (Bengtsson, Werker, et al. 2008; Basak et al. 2011; Wen et al. 2010; Valentino et al. 2015). Bengtsson et al. (2008) observed maximum accumulation with nutrient limitation, 43-48% g PHA/ g total suspended solids (TSS) compared with 32% gPHA/gTSS in excess of nutrients. Wen et al. (2010) obtained in nutrient limiting conditions a maximum PHA accumulation of 59% of the cell dry weight and a productivity of 1.61 mg PHA/ mg COD.

2.5.3.1.2. OXYGEN CONCENTRATION

Tsuge et al. (2002) reported the influence of dissolved oxygen concentration (DO) on PHB accumulation by activated sludge. According to this study, by limiting the concentration of oxygen better yields of PHA storage could be achieved. At low DO concentrations, ATP is mainly used in substrate transportation into the cell and is less available for biomass growth. Moralejo-Gárate et al. 2013 also studied the effect of oxygen supply rate in the production of biopolymers from glycerol, namely PHA and polyglucose. This study showed that oxygen limitation during both community enrichment step and accumulation steps favored polyglucose storage over PHA (Moralejo-Gárate et al. 2013). Similar results were obtain when working with pure cultures (Tsuge 2002; Faccin et al. 2013; Kshirsagar et al. 2012).

2.5.1.3. pH AND TEMPERATURE

Generally, studies on the effect of pH on PHA accumulation report that higher productivities are obtained when same or a slightly higher pH values are used in both selection and accumulation stages. According to what was reviewed by Dias et al (2006) optimal pH values are usually in the range of 8-9, but in the end each culture will perform better at its optimum pH value (Dias et al. 2006).

The influence of temperature is also very dependent on the microbial community and other operational parameters. Optimal values reported can be very distinct, Johnson et al (2010) described maximum production at 30ºC, while 10ºC was the value reported by Chinwetkitvanich et al. (2004). (Johnson et al. 2010; Chinwetkitvanich et al. 2004).

2.6. SUBSTRATE

As said before, substrate is one of the main cost contributors of PHA production and can represent up to 50% of the total cost (Dias et al. 2006). For this reason, the use of cheap substrates is gaining attention. Many agricultural and industrial substrates - like cheese whey, paper mill effluents, sugar cane molasses and olive oil mill effluents - were already studied for acidogenic fermentation toward PHA production (Bengtsson et al. 2008; Beccari et al. 2009; Albuquerque et al. 2007).

In this project, fermented HSSL was used as substrate. HSSL is a by-product of the pulping of *Eucalyptus globulus* wood in paper production processes (Pereira et al. 2012; Xavier et al. 2010). The main carbon sources in this substrate are lignosulphonates (LS) and phenolic derivatives (118 g/L), pentoses – xylose being the predominant sugar (39 g/L) – and acetic acid (16 g/L) according to the data provided from CAIMA. The presence of some compounds reported as microbial inhibitors, such as low molecular weight lignosulphonates, phenolic compounds and furfural, limit the use of HSSL for bioprocessing. However HSSL was already successfully used as substrate for bioetanol and PHA production, showing that the microorganisms are able to adapt themselves to these conditions (Pereira et al. 2013; Queirós et al. 2015).

This type of by-product proved to be valuable raw material and its use can be sustainable in many levels. The use of this type of biomass, derived from waste, to produce valuable intermediate and final products, fuels and energy does not have the ethical implications that biomass from cereal crops have, since they do not compete with the food sector (Carvalheiro et al. 2008; Clark & Deswarte 2008; Kamm et al. 2010). HSSL is a suitable candidate to be integrated in a lignocellulosic-based biorefinery.

By definition, biorefineries are analogous to oil-based refineries, but in which biomass is converted into energy and biomaterials, in a sustainable way. Lignocellulosic-based biorefineries are centered in lignocellulosic materials, such as HSSL. These materials can be converted in various products of added value, such as biofuels like bioethanol, biobutanol or biodiesel, organic acids, polysaccharides and microbial biomass (Karimi 2015).

Therefore, and due to the large amounts of HSSL produced each year (about 1000000 tad), the process described in this work has the potential to be integrated in a biorefinery based in this by-product, and provide an economically and ecologically sustainable way to produce bioplastics.

CHAPTER 3: METHODS AND MATERIALS

3.1. CULTURE

The MMC used in this work was collected from an aerobic tank from the wastewater treatment plant SIMRia - Aveiro Sul, on September of 2015.

3.2. CULTURE MEDIA

Two of the main byproducts of Caima – Indústria de Celulose SA (Constância, Portugal), HSSL from magnesium based acidic sulfite pulping of *Eucalyptus globulus* and Condensate, originated by successive evaporations of the HSSL, were used in this work.

HSSL was submitted to a preliminary treatment. The pre-evaporated HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. To remove part of its most recalcitrant compounds pH was adjusted to 7.0 with 6 M KOH, followed by aeration with compressed air (6 hours per liter of HSSL, 6 h/L). Then, HSSL was centrifuged for 1 h at 5000 rpm and the precipitated colloids were filtered off using a 1.0 µm pore size (VWR 692) (Pereira et al. 2012). The main components of HSSL were LS (≈ 162 g/L) along with xylose and acetic acid (≈ 50 and 18g/L, respectively. No phosphates and ammonia were detected. The HSSL was then submitted to an acidification step. The acidification was performed by an aerobic culture inoculum in a CSTR system. Different conditions were used and originated two different streams with distinct SCOAs profiles, Stream A and Stream B.

3.2.1. STREAM A

Stream A was produced in a CSTR system without pH control, with a HRT of 2.34 days and of 3 days. The average SCOAs profile of Stream A was 4.43/36.7/32.1/26.0/0.86%

of lactate, acetate, propionate, butyrate and valerate, respectively. The acidified effluent was submitted to biomass removal, pH adjustement to 6.5 and dilution with a mineral solution to obtain the desired concentration of SCOA for each period of operation of the SBR. The mineral solution composition was 0.160 g/L of $MgSO_4·7H_2O$, 0.080 g/L of $CaSO_4·2H_2O$, 0.008 g/L of $Na_2MoO_4·2H_2O$, 0.160 g/L of NH_4Cl, 0.4 g/L of CH_4N_2S. The medium was autoclaved for 20 min at 121 ºC, afterwards 0.016 g/L of KH_2PO_4 and 0.064 g/L of K_2HPO_4 were added under sterile conditions. This stream was used in both selection and accumulation steps.

3.2.2. STREAM B

Stream B was produced in a system with pH controlled at 6, with a HRT of 3 days. The average SCOAs profile achieved was 0.01/57.8/27.0/14.8/0.36% of lactate, acetate, propionate, butyrate and valerate, respectively. Stream B was then submitted to the same procedures as Stream A, and was used in both selection and accumulation steps.

3.2.3. CONDENSATE

Condensate was mainly composed by acetate (16.5 g/L), methanol (0.84 g/L) and furfural (0.83 g/L). Before its use, pH was adjusted to 6.5 and it was diluted with a mineral solution to obtain the concentration of SCOAs necessary for each assay. The mineral solution composition was 0.160 g/L of $MgSO_4·7H_2O$, 0.080 g/L of $CaSO_4·2H_2O$, 0.008 g/L of $Na_2MoO_4·2H_2O$, 0.160 g/L of NH_4Cl, 0.4 g/L of CH_4N_2S, 0.016 g/L of KH_2PO_4 and 0.064 g/L of K_2HPO_4. Condensate was only used in the accumulation step.

3.2.4. MATRIX

The matrix is the exhausted medium after SCOAs consumption of the selection reactor. It was withdraw at the end of each cycle and filtered using a 1.0 µm pore size (VWR 692). Then it was supplemented with synthetic SCOAs and used in the accumulation assays.

3.3. REACTOR OPERATION AND SAMPLING

3.3.1. SELECTION SBR

In order to select a PHA-storing culture, the operation of the selection SBR occurred for 180 days. The selection SBR set-up was as shown in Figure 7. The reactor working volume was 1.5 L and worked under aerobic dynamic feeding strategy, during which alternating feast and famine phases were imposed per cycle.

Four periods of operation can be defined according to the selection conditions imposed. For the first 25 days – Period 1 – the cycle length was 24h, comprising 22.5 h of aerobiosis, 1 hour of settling, with agitation and aeration switched off, 0.5 h of withdrawing, half of the reactor volume was removed by a Watson – Marlow Pump 101 F/R and finally, the volume replacement, with fresh medium for period of 15 minutes with Watson – Marlow SCI 400 pump. The hydraulic retention time (HRT) during this period was 2 days. The reactor was fed with 750 mL of Stream A with a SCOAs concentration of 35 Cmmol/L. After this, the cycle length was reduced to 12h – Period 2 – until the 90th day, with 10.5 hours of aerobiosis. The hydraulic retention time in this period was 1 day. Everything else was kept the same. In Period 3 the concentration of SCOAs in the feed was increased to 50 Cmmol/L, keeping the same HRT. Finally after the 156th day of operation, Period 4, the reactor was fed with Stream B. During all the operation period an SRT of 5 days was imposed.

Reactor stirring (400 rpm), aeration and feeding and withdrawing pumps were controlled with timers. Dissolved oxygen and temperature were monitored using Oxygen

meter Transmitter M300 (Mettler-Toledo Thornton, Inc) and pH measured with Crison-PH 28 P.

In order to prevent foam formation, diluted silicone anti-foam (1:20) was manually added when excessive foam was observed. The reactor walls and electrode surfaces of the SBR were cleaned on a daily basis in order to prevent excessive biofilm formation.

FIGURE 7 – Selection SBR set-up

3.3.2. BATCH ACCUMULATION ASSAYS

During this study several accumulation assays were conducted in a batch reactor, BIOSTAT® A Plus – Sartorius, with a working volume of 4 L, without temperature control and sterile conditions. A respirometer was coupled to the bioreactor and constantly stirred at 500 rpm. The medium was circulated by a Watson – Marlow SCI 400 pump and DO registered using Oxygen meter Transmitter M300 (Mettler-Toledo Thornton, Inc). The reactor was stirred at 300 rpm and the values of pH, DO and temperature were monitored. The accumulation reactor set-up was as shown in Figure 8. The inoculum used was collected from the SBR and aerated during 15 h to ensure accumulated PHA consumption. Tests were performed, when the selection SBR achieved a pseudo-stationary state (PSS), by adding feed to the system in a pulse-wise manner, to avoid potential substrate inhibition. Pulses were added when an abrupt increase in the dissolved oxygen was observed. Several tests were conducted under different conditions as seen in Table 5. In the end of the test biomass was collected for PHA extraction and characterization.

3.3.3. SAMPLING

Concerning the selection SBR, several samples were collected in order to monitor the overall performance during the cycles. For each cycle, a sample was collected before the beginning of the feeding (t = 0 h), immediately after the stop of feeding pump (t = 0.25 h), with a 0.5 h interval until t = 3 h, and after that at intervals of 1 h until t = 8 h. At the time of the sample collection the values for pH, temperature and percentage of dissolved oxygen were registered. During kinetic tests samples were collected every 10 minutes.

Samples were centrifuged at 13000 rpm for 10 min (Mettich Zentrifugen MIKRO 120). The solid was separated from the supernatant and both freezed at -16 ºC. The first was used for PHA determination and the later for determination of pH, SCOA, LS, ammonium and COD.

FIGURE 8 – Accumulation reactor set-up.

TABLE 5 – Conditions of each accumulation assay.

Assay	Substrate	$Cmmol_{SCOA}$/ pulse	Nº of Pulses	Time (h)	Limitation
AT1	Stream A	35	5	2.5	None
AT2	Stream A	35	5	8	Nitrogen
AT3	Condensate	35	4	4.5	None
AT4	Condensate	35	5	5	Nitrogen
AT5	Matrix + Acetate	35	5	4	None
AT6	Matrix + Propionate	35	4	8	None
AT7	Stream A	50	9	12	Nitrogen
AT8	Stream B	50	8	12	Nitrogen
AT9	Condensate	50	9	9.5	Nitrogen

3.4. PHA EXTRACTION

In order to extract PHA from the accumulation tests the cell suspension was centrifuged at 5000 rpm for 30 minutes at 4 ºC. The supernatant was discarded and the cells were washed with a 0.9% NaCl solution. This procedure was repeated several times to ensure the biomass was clean and then was lyophilized. In a covered glass, 30 mL of chloroform were added per gram of biomass and left to incubate at room temperature, 200 rpm for 24h. By the end of this time the cellular residues were filtered (VWR Glass microfiber filter 629 with a pore diameter of 1 µm) and discarded. After chloroform evaporation, the PHA film extracted was collected for posterior analysis.

3.5. ANALYTICAL METHODS

3.5.1. AMMONIUM QUANTIFICATION

The ammonium concentration was followed using a Thermo Scientific Ion Selective Electrode. To 1 mL of the samples 20 µL of Ionic Strength Adjuster (ISA) were added. This solution is composed of 5 M NaOH, 0.05 M EDTA and 10 % methanol. The calibration of the electrode was done resorting to a standard curve (0-5 mM).

3.5.2. CARBON SOURCE ANALYSIS

SCOA concentration evolution was measured by HPLC. 600 µL of the samples were acidified with 50 µL of 0.25 M H_2SO_4, filtered using a membrane of 0.2 µm (Whatman) at 8000 rpm (Mini Spin Eppendorf) for 20 minutes and injected in an Aminex HPX-87 H column (Bio-Rad Laboratories, CA, USA), at 60 ºC, and a refractive index detector (Merck, Germany), using H_2SO_4 0.01 N as eluent (0.5 mL/min). A calibration curve using was done with synthetic acids (0-5 g/L).

3.5.3. VOLATILE SUSPENDED SOLIDS

The amount of biomass in both reactors was quantified as volatile suspended solids, according to Standard Methods (Clesceri et al. 1999). Samples of 5 mL were collected during the monitored cycles and accumulation batch tests. Then, samples were filtrated using previously dried and weighted filters (VWR Glass microfiber filter 629 with a pore diameter of 1 μm) with vacuum filtration. The membranes were placed in the oven at 105 °C for 24 hours. After cooling down they were weighted and the biomass concentration was determined in g/L of total suspended solids (TSS). Afterwards the membranes were submitted to 550 ºC for 2 h and weighted after cooling to determine VSS in g/L.

3.5.4. CHEMICAL OXYGEN DEMAND

COD was measured with Spectroquant Kit (Merck) and the solutions used were prepared according to Standard Methods: a digestive aqueous solution with $K_2Cr_2O_7$, $HgSO_4$ and H_2SO_4 and an acid solution with H_2SO_4 and $AgSO_4$. To 2 mL of sample properly diluted were added 1.2 mL of digestive solution and 2.8 mL of acid solution. The mixture was incubated at 150 ºC for 2 h. After cooling, the absorbance was measured. The calibration was done with glucose with COD concentrations between 0 – 1 g/L.

3.5.5. LIGNOSULPHONATES QUANTIFICATION

The monitorization of lignosulphonates (LS) content was performed according to Restolho et al. (2009). The measurement was performed using a UV Spectrophotometer (Shimadzu UVmini-1240) at 275 nm, after a dilution of 1:10. The lignosulphonates concentration was calculated resorting to the Beer-Lambert law, using a ε = 7.41 $g^{-1}cm^{-1}$ (Xavier et al. 2010).

3.5.6. PHA QUANTIFICATION

The determination of PHA cell content was determined using GC according to Moita & Lemos (2012). The pellet collected after the centrifugation of 1.5 mL of sample was lyophilized. The biomass was incubated with 1:1 solutions of chloroform with heptadecane as internal standard and an acidic methanol solution (20% H_2SO_4), at 100 ºC for 3.5 h. After cooling, 0.5 mL of water was used for extraction. The chloroform phase was collected and molecular *sieves* (0.3 mm) were added to ensure water adsorption. 2 µL of the obtained solution were injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID, Konik Instruments HRGC-3000C). A ZBWax-Plus column was used with hydrogen as the carrier gas (50 kPa) as well as split injection at 280 ºC with a split ratio of 1:6. The oven temperature program was as follows: 60 ºC; then 20 ºC/min until 100 ºC; then 3 ºC/min until 175 ºC; and finally 20 ºC/min until 220 ºC. The detector temperature was set at 250 ºC. Hydroxybutyrate and hydroxyvalerate concentrations were calculated using standards of a commercial P(HB-HV) (88%/12%, Aldrich) and corrected using a heptadecane internal standard.

3.6 CALCULATIONS

PHAs content was calculated as a percentage of TSS on a mass basis:

$$\% \text{ PHAs} = \text{gHA}/\text{gTSS} \times 100$$

Feast to famine ratio (F/F) was calculated dividing the time needed to the consumption of SCOAs by the remaining time of the cycle.

SCOAs volumetric and specific consumption rates (-qSCOAs), acetate volumetric consumption rate (–qAcet), propionate volumetric consumption rate (–qProp), butyrate volumetric consumption rate (–qBut), PHA volumetric and specific production rates (qPHA), PHB volumetric production rates (qPHB), PHV volumetric production rates (qPHV) were determined by adjusting linear functions to the experimental data for each variable concentration over time, and calculating the first derivative at time zero. In case of specific rates each variable was divided by the biomass concentration at that point.

The oxygen uptake rate (OUR) was determined by adjusting linear functions to the experimental data from the respirometer over time, and calculating the first derivative at time zero. The %DO was previously converted to mgO2/L.s considering an oxygen saturation concentration at 28 ºC of 7.36 mgO2/L.s (Queirós et al. 2014). Endogenous OUR was determined before the addition of the substrate using the same method (Garcia-Ochoa et al. 2010).

PHA production yield on substrate (YPHA/S) was calculated by dividing the amount of PHA by the total amount of SCOAS consumed. PHA specific productivity was calculated dividing the amount of produced PHA by biomass and time and the volumetric productivity dividing the amount of produced PHA by volume and time.

CHAPTER 4: RESULTS AND DISCUSSION

4.2. SELECTION STEP

4.2.1. SBR OPERATION

The SBR was operated for 180 days and the culture selection was achieved by imposing ADF conditions. During the operational period, several parameters were monitored: SCOAs uptake; PHA production; biomass concentration; F/F ratio; COD and ammonium uptake. Those were used to evaluate the adaptation of the culture to the imposed conditions and its capacity to accumulate PHA as well as the selective pressure conditions of the SBR reactor.

As discussed previously in Chapter 2, the selection step must be a compromise between PHA accumulation capacity and biomass growth, since the volumetric productivity of the accumulation step is dependent not only on the ability of the MMC to accumulate PHA but also on cell density (Reis et al. 2011). Biomass growth and PHA production are processes that compete for the carbon source (Serafim et al. 2008), so OLR manipulation is a key factor in the process. In this case OLR was kept low in the beginning of the process to ensure the adaptation of the culture to the medium and was gradually increased, when the culture seemed to achieve an apparent stationary phase, to promote an increase biomass concentration. For this reason, during the operational period, parameters, such as cycle duration and OLR, were altered in order to raise selective pressure. Thus three periods of operation can be distinct: 24 h cycles with an OLR of 2.2 gCOD/L.d from the start until day 26; 12 h cycles with an OLR of 4.5 gCOD/L.d from day 26 until day 90 and finally 12 h cycles with an OLR of 7.0 gCOD/L.d from day 91 until day 156. After the 156th day the substrate was changed from Stream A to Stream B (OLR of 6.7 gCOD/L.d) so that the capacity of the MMC to adjust to a fermented stream with a different SCOAs profile could be evaluated. This stream was used until the end of this project. Table 6 summarizes the conditions used in each period of operation of the SBR for a better understanding.

TABLE 6 - Conditions imposed to the SBR in each period of operation

Period Designation	Cycle Duration (h)	OLR (gCOD/L.d)	SCOAs (Cmmol/L)	Feeding	SRT (days)
P1	24	2.2	17.5	Stream A	5
P2	12	4.5	35	Stream A	5
P3	12	7.0	50	Stream A	5
P4	12	6.7	50	Stream B	5

Figure 9 shows the variation of F/F ratio, biomass concentration, SCOAs uptake and PHA production throughout the operational time.

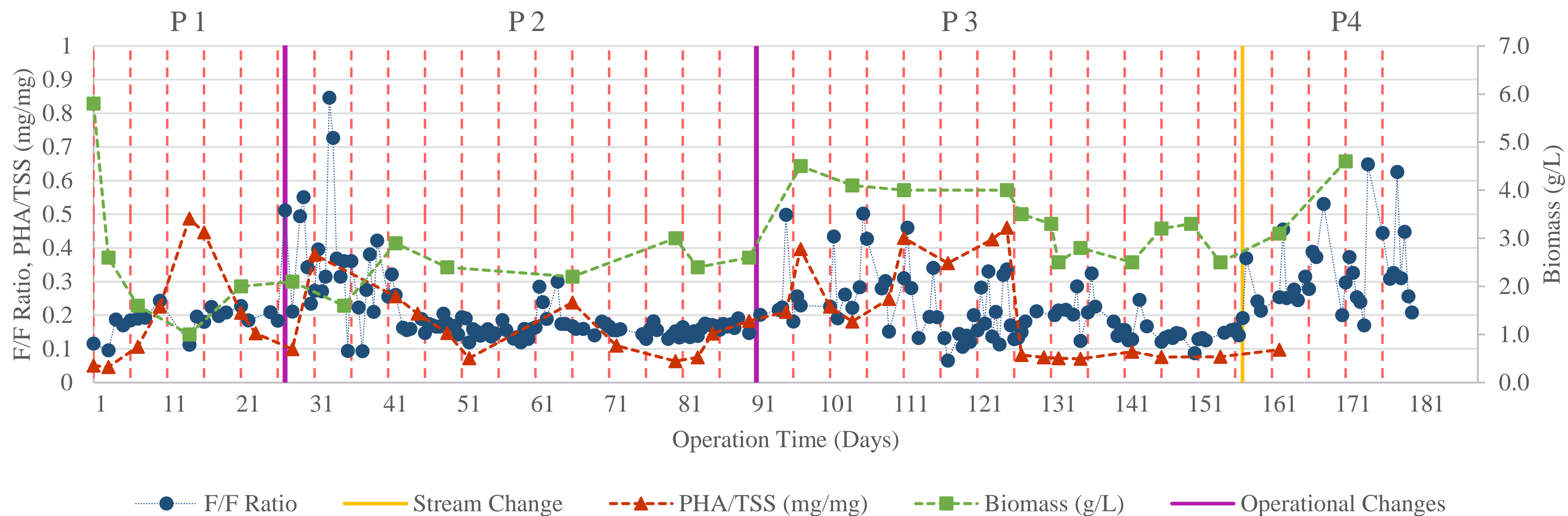


FIGURE 9 – SBR Performance: Variation of the F/F Ratio, Biomass concentration (g/L) and PHA/TSS (mg/mg) over the operational periods

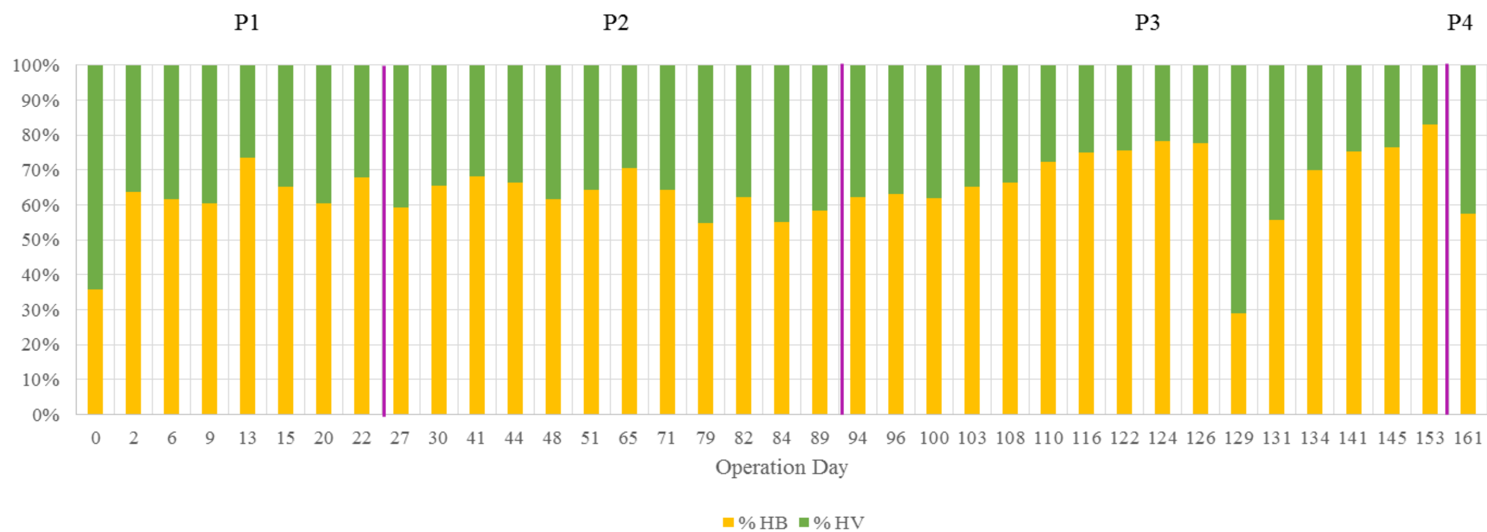


FIGURE 10 – Monomer composition through the operational periods

4.2.1.1. PERIOD 1 - 24H CYCLE, 17.5 CmmolSCOAS/L, STREAM A

Period 1 (P1) corresponds to the operation with 24h cycles and a OLR_{SCOAs} of 17.5 CmmolSCOAs/L, from the first day to the 26th.

The F/F ratio variation throughout the operational time, represented in Figure 9, is being used as an indicator of culture adaptation and consequent PHA storage capacity (Valentino et al. 2014; Reis et al. 2011). Since the exhaustion of the carbon source typically leads to an abrupt increase in the DO in the medium, this parameter can be used to practically identify the transition between the feast and famine phases and so calculate the F/F ratio (Queirós et al. 2015). As previously discussed in Chapter 2, a correlation between this ratio and the PHA accumulation ability was established, in several studies. Cycles with feast phases under 20-25% of the total cycle duration were associated with good storage response. On the other hand in longer feast phases storage played a negligible role and the substrate was diverted mainly towards growth (Dionisi et al. 2006a; Valentino et al. 2014). During P1, F/F ratio was low for the first 3 days and then stabilized with an average value of 0.19 without major variation.

Biomass concentration in the selection reactor is a relevant parameter since it will serve as an indicator of the adaptability of the MMC and because of its influence the productivity on the PHA accumulation step and great impact in the overall process (Oehmen et al. 2014). The evolution of this parameter in the selection SBR over time is represented in Figure 9. The initial inoculum had a biomass concentration of 5.8 gVSS/L. The selective pressure imposed reduced the concentration to 1.6 gVSS/L after 6 days. This could be the result of the elimination of several microorganisms that were not able to reproduce themselves in the imposed conditions, due to the inability to consume SCOAs, accumulate PHA or to inhibition by compounds present on the substrate. After this initial period the biomass concentration in the reactor remained constant with an average value of 1.7 ± 0.4 gVSS/L, indicating culture stability.

PHA cell content represented in the Figure 9 corresponds to maximum value of PHA produced per TSS during each monitored cycle. In the beginning of the operation PHA content was very low, 5% cdw, and it increased over time having reached a peak on the 13th

day, 50% cdw. This value was the highest not only for period with 24h cycles but also for the overall operation. The PHA content then decreased to 14% cdw on the 22nd day.

The monomer composition of the PHA accumulated during the SBR operation was also evaluated and it is described in Figure 10. The results showed that, during all the operation time, a copolymer of P(HB-co-HV) was produced, which was expected since the carbon source had precursors for both these monomers (Lemos et al. 2006). During P1, HB was the predominant monomer and its percentage in the polymer remained fairly constant especially after stabilization (6th day), with an average of 65% of HB.

Considering all the parameters analyzed a pseudo stationary state could be established from day 9 to day 25, which corresponded to an adaptation period of 9 days.

4.2.1.2. PERIOD 2 - 12H CYCLE, 35 CmmolSCOAS/L, STREAM A

Period 2 (P2) corresponded to the operation after the change of cycle duration to 12 h cycles and an OLR_{SCOAs} of 35 CmmolSCOAs/L, from the first day to the 26th.

The evolution of all parameters is shown in Figure 9. The change in the cycle duration led to a period of F/F ratio instability for about 15 days, with values ranging from 0.09 to 0.85. Since the 42nd day of operation to the 89th, F/F ratio decreased and remained around 0.16. This value represents a good indicator of the MMC's PHA accumulation capacity (Reis et al. 2011).

Biomass concentration in the SBR experienced a slight increase after the change. This was not observed until the 41st day, which might be related to the culture adaptation to the new conditions. Afterwards the average value of biomass concentration was 2.6 ± 0.3 gVSS/L.

An increase of polymer content to 38% cdw on the 30th day of operation was also observed in P2. Then the values of PHA content remained between 26% cdw and 6% cdw for the rest of the operational time, with an average concentration of 15% cdw. In the cycles monitored before the change of the OLR there was a slight increase of the PHA content.

There was no visible impact in the polymer composition, nonetheless after the 44th day there was a small decrease in the HB content to an average of 62%, as seen in Figure 10.

A PSS could be identified from the 42nd day until the operational conditions were changed again, since all parameters monitored remained constant during this time. This means that the culture took 17 days to adapt to the new operational conditions.

4.2.1.3. PERIOD 3 - 12H CYCLE, 50 CmmolSCOAS/L, STREAM A

On the 90th day, OLR_{SCOAs} increased to 50 CmmolSCOAs/L, corresponding to Period 3 (P3) of operation. This led to another stage of adaptation to the new selection conditions.

Figure 9 shows the evolution of the monitored parameters. F/F ratio was unstable for 32 days, with variations between 0.07 and 0.50, and then the values became more constant. From the 122nd day till the 155th a slow decrease of the F/F ratio values, from 0.20 to about 0.15, was observed and it was an indication of the gradual adaptation to the selective pressure.

After the increase in the OLR there was an increase of biomass concentration in the reactor. For the first 34 days biomass concentration was around 4.2 ± 0.2 gVSS/L, but for the remaining time under this condition there was a decrease to 3.0 ± 0.4 gVSS/L. This decrease occurred after the apparent stabilization of the reactor that can be inferred by the other analysed parameters and could be explained due to the decrease of the PHA content in the cells that happens in the same period, as observed in other studies (Villano et al. 2014).

At the beginning of P3 the values of the accumulated polymer in the cells displayed inconsistency, ranging within 18-46% cdw, such can be due to the fact that the culture was still adapting to the new conditions. From day 126 onwards the PHA content remained constant at 8% cdw with little variation (7-10% cdw). Regarding the polymer content, illustrated in Figure 10, an increase of the HB content was observed, from 62% to 78%, and followed by a slight prevalence of HV. This increase of the HV content happened after the stabilization of the other parameters monitored and could be explained by a different composition in the feed for that cycle, with a higher propionate and valerate content. After that HB content rapidly increased to previous values, having achieved its highest percentage on the 153rd day, 83%.

The data collected is consistent and indicates that the culture had reached a PSS from the 126th day to the 155th, corresponding to an adaptation period of 36 days.

4.2.1.4. PERIOD 4 - 12H CYCLE, 50 CmmolSCOAS/L, STREAM B

On the 156th day, a stream with a different SCOAs composition started to be fed to the SBR, corresponding to Period 4 (P4).

This change was followed by a period of instability, with F/F ratio values ranging from 0.17 to 0.65. Since the reactor was operated for only 23 days after the stream change, a stabilization of the F/F ratio value was not observed. Although it was expected to happen if the SBR continued to operate. An increase in the biomass concentration was also observed, but the data available is insufficient to draw any major conclusions. All the parameters are illustrated in Figure 9.

Due to logistics and time constrains the PHA content for the cycles monitored conducted after the stream change was only determined for one cycle and therefore no tendencies could be observed. Nonetheless this sample also showed the presence of a copolymer of P(HB-co-HV), as seen in Figure 10.

Considering all this is safe to say that the SBR did not reach a PSS for P4, and its operation should have been continued.

4.2.1.5. OVERALL OPERATION

Considering the variation of each parameter during all the operational time some conclusion can be taken. After each change of conditions there was a clear period of instability, with variations in the F/F ratio, followed by a stabilization with values around 0.10-0.20, values that are clearly related to a storage response (Albuquerque et al. 2010; Reis et al. 2011). This pattern was observed in other studies and can act as an indicator of the

culture adaptation (Valentino et al. 2015; Villano et al. 2014). The ratio values after stabilization go according to those obtained in previous works with HSSL (Queirós et al. 2015).

The obtained values are coherent with those found on literature, since several works using ADF as a selection strategy showed good accumulation rates for ratios under 0.2. Dionisi et al. (2006) tested the effect of the OLR on the performance of an SBR fed with a mixture of SCOAs and reported ratios under 0.25 for good accumulation capacity. Johnson et al. (2009) obtained very low F/F ratios (0.1) when the selective pressure imposed on the culture selection SBR fed with acetate was maximised. Pilot scale SBR for treating filtered influent municipal wastewater also had ratios of approximately 0.13. Also using a complex substrate, sugar molasses, Morgan-Sagastume et al. (2015) obtain F/F ratios of 0.21. Ben et al. (2016) reported ratios of around 0.15 for an SBR fed with fed fermented brewery wastewater.

Considering the biomass in the SBR it can be observed that overall there was an increase all through the operational time. Although the average value of active biomass in the last stable phase of P3, 3.0 gVSS/L, was higher than that obtained by Queirós et al. (2014) using HSSL as carbon source, 0.98 gVSS/L, it was not significantly superior to the 2.85 gVSS/L obtained in their latter work (Queirós, Fonseca, et al. 2015). However the organic load used in this work, OLR of 7 for P3 with SCOAs concentration of 1.5 g/L, was significantly lower to the one used by Queirós et al. (2015), OLR of 17 gCOD/L.d and acetate concentration of 1 g/L. This shows that in terms of conversion of substrate into biomass this work had the better performance.

The concentration achieved in this work is similar to those overserved in works with MMC. Serafim et al. (2008) operated three SBRs with different conditions to study its influence in the polymer characteristics and reported that in all the systems the biomass concentration was between 2 and 3 g VSS/L. Using brewery wastewater to select a PHA accumulating culture, Ben et al. (2016) obtained a biomass concentration of 3.2 gVSS/L. Some works however reported higher biomass concentrations in similar systems. The work of Albuquerque et al. (2010) studied the effect of the influent substrate concentration on culture selection, using a complex feedstock as SCOAs source, and while no direct correlation between OLR and VSS was described, the highest biomass concentration

obtained was 5.1 g/L from the PHA-accumulating selection SBR fed with a substrate concentration of 47 Cmmol SCOAs/L. Oehmen et al. (2014) used pH control as a strategy to increase the biomass growth rate while maintaining the specificity of the enrichment for PHA-producing organisms and reported biomass concentrations up to 8 gVSS/L with pH control at 8.

Globally the results showed that for this work when the culture reached a stable state the PHA content was around 12% cdw, usually preceded by an instability phase where the highest PHA content values where observed. Similar behaviour was reported in other studies (Villano et al. 2014). It should be noted that regardless of the imposed conditions PHA content tended to the same values after culture adaptation, around 10% cdw.

Previous works with the same feedstock reported both higher and lower polymer contents. Queirós et al. (2014) reported an average value of 54.2% cdw, while Queirós et al. (2015) observed 4.3% cdw, using HSSL that did not undergo a process of acidification. Several papers on MMC selection for PHA production shown similar polymer contents to those obtained in this study, although literature shows much diversified results. Using bio-oil, Moita & Lemos (2012) reported a maximum PHA content of 9.2% cdw. Villano et al. (2014) described an average concentration around 15% cdw for the period of stable operation of an SBR reactor fed with acetate and propionate mixture. Lower contents were also observed, Morgan-Sagastume et al. (2015) operated a pilot scale reactor integrated in a municipal wastewater treatment plant that selected a culture with biomass PHA contents below 4% cdw. On the other hand, several papers also described higher values, Albuquerque et al. (2010) showed an average maximum PHA content of 25% cdw with sugar molasses as carbon source and Chen et al. (2016) operated four SBRs with different SRT fed with fermented sugar cane wastewater and reported PHA contents between 9.55 and 30.75% cdw.

Altogether the data revealed that the culture was able to produce a copolymer from both Streams fed and regardless of the imposed conditions, with an average composition of 64% of HB and 36% of HV. As expected HB was the predominant monomer, since the main SCOA in the fermented HSSL was acetate, a precursor for HB. The changes in the monomer percentages can be explained by the changes of the SCOAs profile in the feed. Since the fermented HSSL was produced in an ongoing work the relative percentages of each acid in

the effluent produced suffer some changes through time and that can have an impact in the monomer production in the SBR.

P(HB-co-HV) production in selection SBRs using real feedstocks is widely documented (Ben et al. 2016; Albuquerque et al. 2010; Lemos et al. 2006). Similar monomer proportions to those of this study were reported by Moita & Lemos (2012) using bio-oil and Oehmen et al. (2014) with fermented molasses without pH control. Queirós et al. (2015) obtained a copolymer of P(HB-co-HV) with 20% of HV using non fermented HSSL. The higher HV fraction obtained could be the result of the introduction of a pre fermentation step. This resulted in a wider variety of SCOAs or a higher production of organic acids precursors of HV production, like propionate and valerate (Reis et al. 2011).

Resuming, overall three PSSs were identified during the operational time: the first from day 9 to day 25, the second from the 42nd to the 90th day, and finally from the 126th to the 155th day. These steady states are defined, and can be identified in most of the parameters analysed and are related to high selective pressure for PHA storage. During this phases, F/F ratio, PHA content and biomass concentration remained fairly constant and most of the variations could be explained by variations in the SCOAs profile fed to the SBR, pH or temperature, which were not controlled. The adaptation periods, 9, 17 and 36 days respectively, were similar (Albuquerque et al. 2010; Oehmen et al. 2014) or lower to those reported in literature (Ben et al. 2016; Moita & Lemos 2012). Using non acidified HSSL, Queirós et al. (2015) reported an adaptation phase of 270 days and Queirós et al. (2014) did not reach steady-state, this may show that the separate acidification step used in this work could also help in the removal of toxic compounds and favour MMC adaptation.

In this work, the performance of the SBR was fairly constant even without pH or temperature control. During the entire operation temperature varied between 13 and 27 ºC and during each cycle monitored temperature increased an average of 4ºC. The pH of the feed was adjusted to 7 and during the cycles it could increase until 9 due to SCOAs consumption. None of these variations seemed to influence the growth rate or the consumption rate of the substrates by the culture. Such is a major advantage of this system, since temperature and pH control at a larger scale would be a major contribution for the increase of the production costs (Serafim et al. 2008).

The SBR operation met the two main goals for the selection step: an MMC with high PHA storage capacity and biomass concentration. Although the PHA content detected in the SBR in the steady state is low (about 12% cdw) that did not translate to bad accumulation performance as it is described in Section 4.3. Biomass concentration increased with the increase of OLR, however it was not significantly higher than in previous studies (Queirós, Fonseca, et al. 2015). This shows that there is room for improvement, the OLR could have been increase even more, since there seemed to have been no inhibition by the toxic compounds present in HSSL, such as gallic acid, pyrogallol and furfural (Pereira et al. 2013). Other strategies for the promotion of biomass growth could also be introduced, such as pH control as reported by Oehmen et al. (2014).

Regarding the results obtained, it can be considered that the selection step was successful. The MMC was able to subsist and adapt to the HSSL, a complex and toxic substract (Pereira et al. (2013)), and the culture responded positively to the imposed conditions. By the end of the operational time a culture with good capacity for PHA storage had been selected and performed well in the accumulation steps.

4.2.2. CYCLE ANALISYS

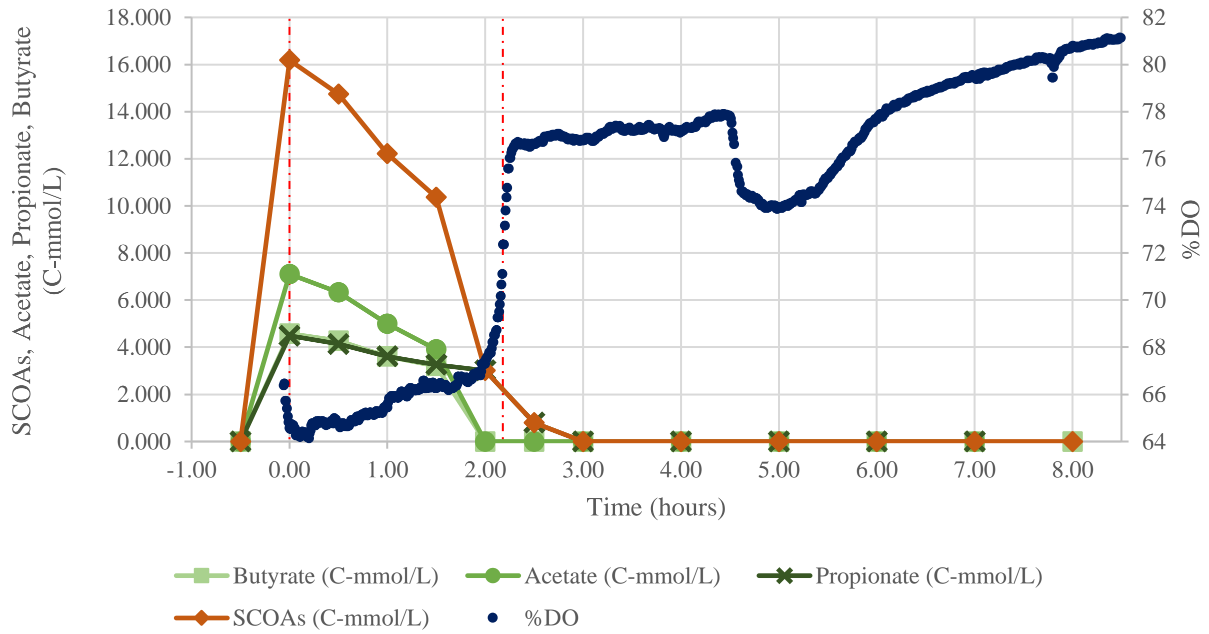
As mentioned before, periodical cycles were analyzed to evaluate the MMC performance. Several parameters were followed, namely concentrations of SCOAs, PHA, biomass, COD, ammonium and LS as well as DO percentage and pH. In section 4.1.1, the performance of the selected MMC was evaluated throughout the SBR operational period in order to assess the efficiency of the selection step. This evaluation should be completed by analyzing representative cycles of the SBR. Table 7 summarizes all the kinetic and stoichiometric parameters determined for each cycle representative of each period of operation.

4.2.2.1. PERIOD 1 – 13TH DAY OF OPERATION (24H CYCLE, 17.5 Cmmmol SCOAS/L, STREAM A)

Figure 11 shows the variation of the several parameters monitored during a cycle in the 13th day of operation, which corresponded to the stable phase of Period 1. For this cycle in particular, SCOAs composition in the fed was 60.1/20.5/11.0/6.4/1.5 % of acetate, propionate, butyrate, lactate and valerate, respectively. The last two however are not represented since they were present in low concentrations and were not detected.

Figure 11 A shows the evolution of the different SCOAs along the cycle. The acids were not consumed at the same rate. Acetate was clearly preferred and consumed at a higher volumetric rate, 3.41 CmmolAcet/L.h, until 2 h. Propionate and butyrate were consumed at lower rates, 1.51 CmmolProp/L.h and 2.10 CmmolBut/L.h, respectively. And their exhaustion in the medium occurred at 2 h and 3 h, respectively. This data, along with the %DO, can be used to determinate the F/F ratio for this cycle. The feast phase begins at 0 h, when SCOAs concentration is at its highest value and there is a decrease in the % DO in the medium. The end of the feast phase corresponds to the increase in the % DO and exhaustion of the SCOAs in the medium, around 2 h. Although there is a small increase in the %DO at 5 h it is not significant to be considered as part of the feast phase and can be associated to the consumption of other components of the HSSL. This makes the F/F ratio for this cycle of 0.09.

A



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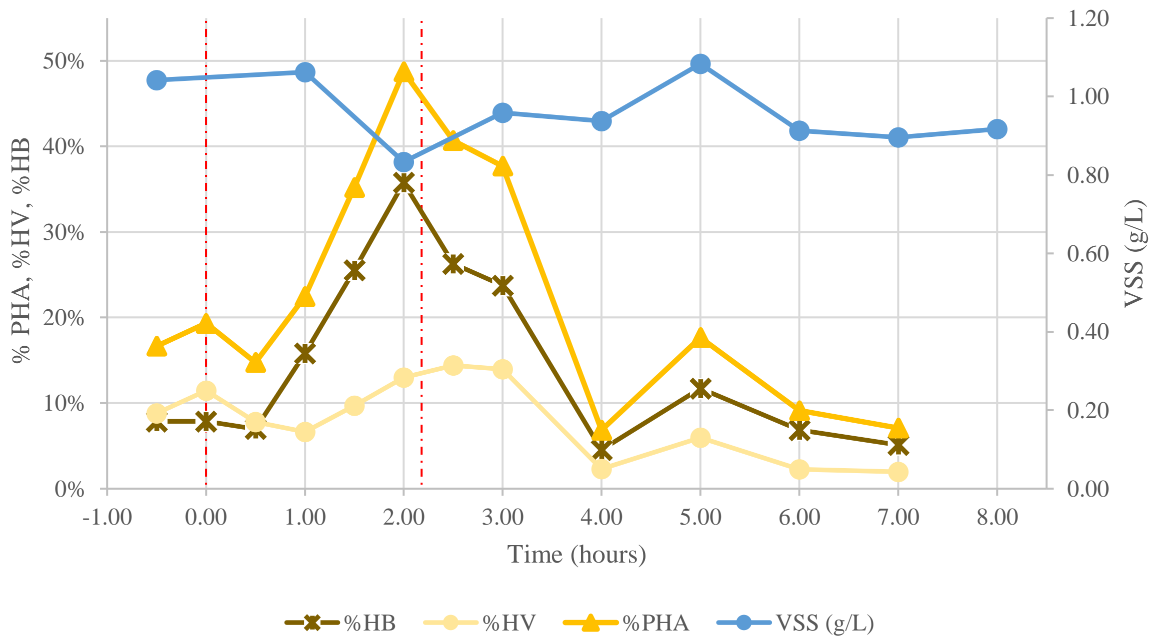


FIGURE 11 - Period 1 – 13th day of operation (24h cycle, 17.5 CmmolSCOAs/L, Stream A)

The variation of PHA monomers through the cycle, represented in Figure 11 B showed that PHB and PHV had distinct tendencies. PHB reached its maximum (36% cdw) at 2 h (volumetric production rate of 0.15 CmmolHB/L.h), that also corresponded to the exhaustion of acetate, the main precursor of HB. HV was produced at a slower rate (0.02 CmmolHV/L.h) as expected, since its precursor propionate was also consumed at a slower rate. The maximum HV, 14% cdw, was verified at 2.5 h, and the production of PHV stopped after propionate exhaustion at 3 h.

The overall PHA production had its maximum (49% cdw) at 2 h, with a PHA production rate of 0.16 CmmolPHA/L.h and an overall rate of SCOAs consumption of 6.27 CmmolSCOAs/L.h, as shown in Figure 11 A. PHA content decreased until 4 h, and then raised again to 17.7% cdw at 5 h. After that the value decreased until the end of the monitored time. Figure 11 A shows the variation of the total SCOAs and % DO in the medium. DO concentration in the SBR is associated with the carbon source consumption (Moita & Lemos 2012; Queirós et al. 2014; Jiang et al. 2012). The decrease of the oxygen concentration to values close to zero after the feeding was due to the high demand of oxygen for SCOAs degradation, as observed at 0 h. When the carbon source is exhausted an abrupt increase of the %DO is usually observed. In this case a clear increase happened at 2.18 h and another after 5 hours of operation. This could be a reaction to the depletion of other carbon sources present in the substrate.

Finally, Figure 11 illustrates the variation of some of other parameters followed during the cycle, namely pH, biomass concentration, temperature, LS concentration, and COD. After the feeding pH decreased from 9.04 to 8.58, and slowly increased with the SCOAs consumption, reaching 8.92 by the end of the monitored period. Biomass concentration showed no particular tendency and varied around 1 gVSS/L. Nonetheless, growth in the feast phase was expected to be observed. In the monitored time there was no evidence of LS consumption, with values remaining at 3 g/L. Ammonium concentration was not measured for this cycle.

4.2.2.2. PERIOD 2 – 48TH DAY OF OPERATION (12H CYCLE, 35 Cmmol SCOAS/L, STREAM A)

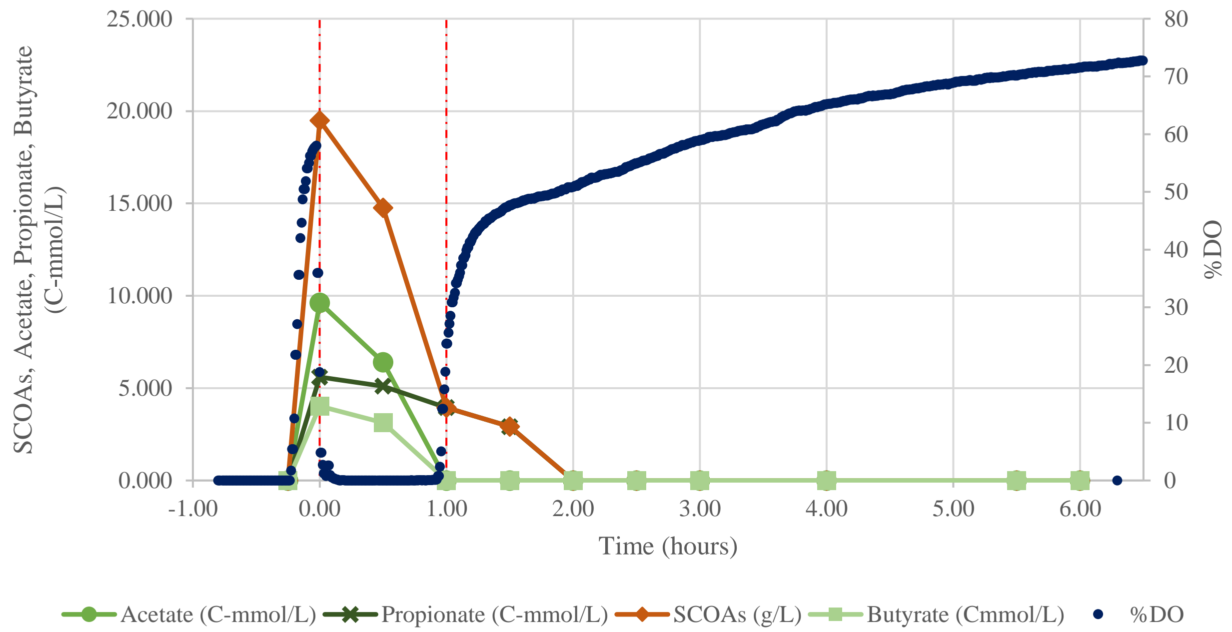
The cycle represented in Figure 12 describes the variation of the monitored parameters on the 48th day of operation, during the PSS of Period 2. The SCOAs composition of the feed in this cycle was 59.8/16.7/13.8/8.2/1.5 % acetate, propionate, butyrate, lactate and valerate. The last two however are not represented since their concentration in the SBR samples was very low and non-detectable.

Figure 12 A shows the consumption of each SCOAs over time. Acetate was still the preferred acid, consumed at the highest rate (9.55 CmmolAcet/L.h) and at a higher value than in cycles from P1, suggesting a better adaptation from the culture. Propionate and butyrate were also consumed at slightly higher rates than in P1, 2.66 and 3.98 Cmmol/L.h, respectively. The exhaustion of the majority of the acids occurred at 1 h.

Figure 12 B also shows the variation of PHA monomers accumulated in the cells. Both monomers presented similar behaviours, increasing until 1 h and then decreasing during the monitored time. PHB maximum content was 9% cdw, produced at a rate of 0.07 CmmolHB/L.h and PHV maximum content was 6% cdw produced at a rate of 0.05 CmmolHV/L.h. As before, PHB was produced at a higher rate. The results of both accumulation content and production rates are inferior to those of the P1 cycle.

In Figure 12 B, the overall PHA maximum is represented (15% cdw), produced with a rate of 0.07 CmmolHA/L.h at 1 h. Figure 12 A also shows that SCOAs were mainly consumed during the first hour of the cycle (10.08 CmmolSCOAs/L.h), although there was a small consumption after this period. Regarding %DO concentration the profile is very clear, with a decrease right after the feeding to 0% and a sudden increase at 1 h. This is associated with the end of the feast phase. Therefore we can clearly define feast and famine phases, and calculate an F/F rate of 0.083.

A



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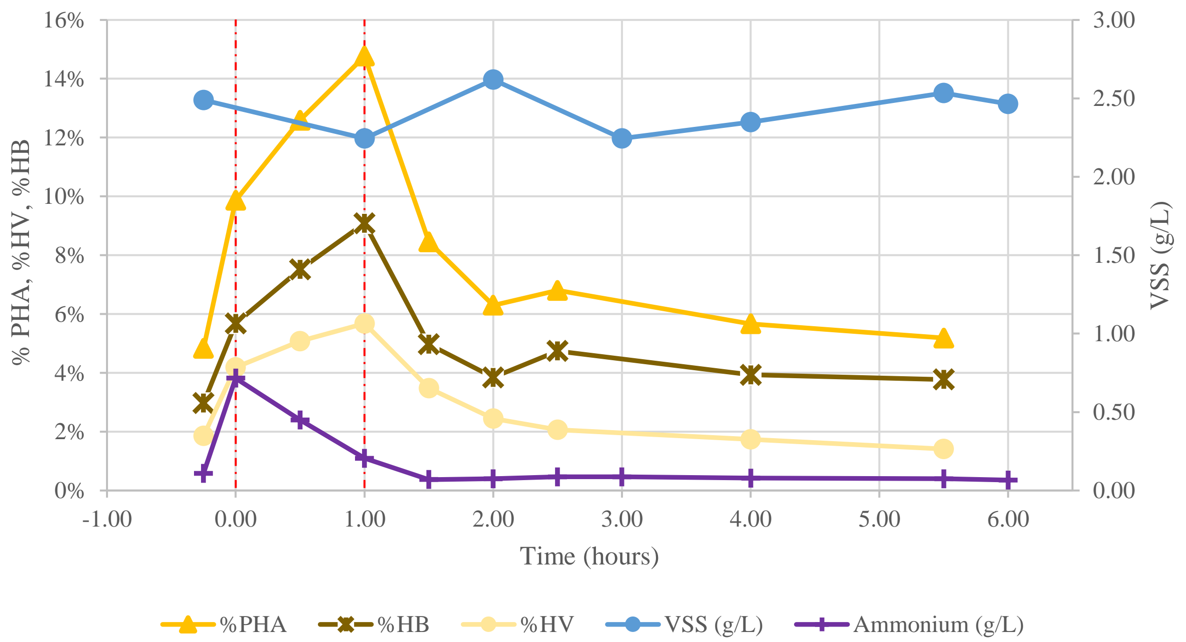

FIGURE 12 - Period 2 – 48th day of operation (12h cycle, 35 CmmolSCOAs/L, Stream A)

Other parameters followed are illustrated in Figure 12. Biomass concentration remained around 2.42 gVSS/L with no major variations. Ammonium concentration had the expected behaviour decreasing during the cycle until its depletion at 1.5 h. This was expected since ammonium consumption is associated to cell growth (Johnson, Kleerebezem & Loosdrecht 2010). The pH as the same behaviour as in the previous cycle, decreasing after feeding and then increasing till the end of the cycle (from 8.55 at -0.25 h, to 8.32 at 0 h, and then to 8.72 at 6 h). Lignosulphonates concentration was 3.8 g/L and did not change during the monitored time, indicating that, like in the previous cycle, they were not consumed.

4.2.2.3. PERIOD 3 – 141ST DAY OF OPERATION (12H CYCLE, 50 Cmmol SCOAS/L, STREAM A)

Figure 13 illustrates the variation of several parameters monitored during a cycle on the 141st day of operation, which corresponded to the stable phase of Period 3. For this cycle the SCOAs composition of the fed was 57.4/29.2/9.5/0.7/3.2 % of acetate, propionate, butyrate, lactate and valerate, respectively. However only the first two were at detectable concentrations in the method used and represented.

Figure 13 A shows SCOAs evolution along the cycle. The acids reached their maximum concentration on the beginning of the cycle and were consumed at different volumetric rates, acetate at 6.48 CmmolAcet/L.h and propionate at 3.97 CmmolProp/L.h, with acetic acid as the preferred carbon source. Both acids were consumed until 1.5 h. Figure 13 B shows that the maximum PHB (7% cdw, production rate of 0.026 CmmolHB/L.h) was produced at 1.5 h, when SCOAs exhaustion was observed.

Figure 13 summarizes the more relevant parameters monitored and allows to see a clear distinction between the feast and famine phases. %DO concentration decreased to null values after the beginning of the feast phase at 0 h, and increase drastically to 80% at 1.5 h. Also at this time the maximum of PHA content was verified (9% cdw with a production rate

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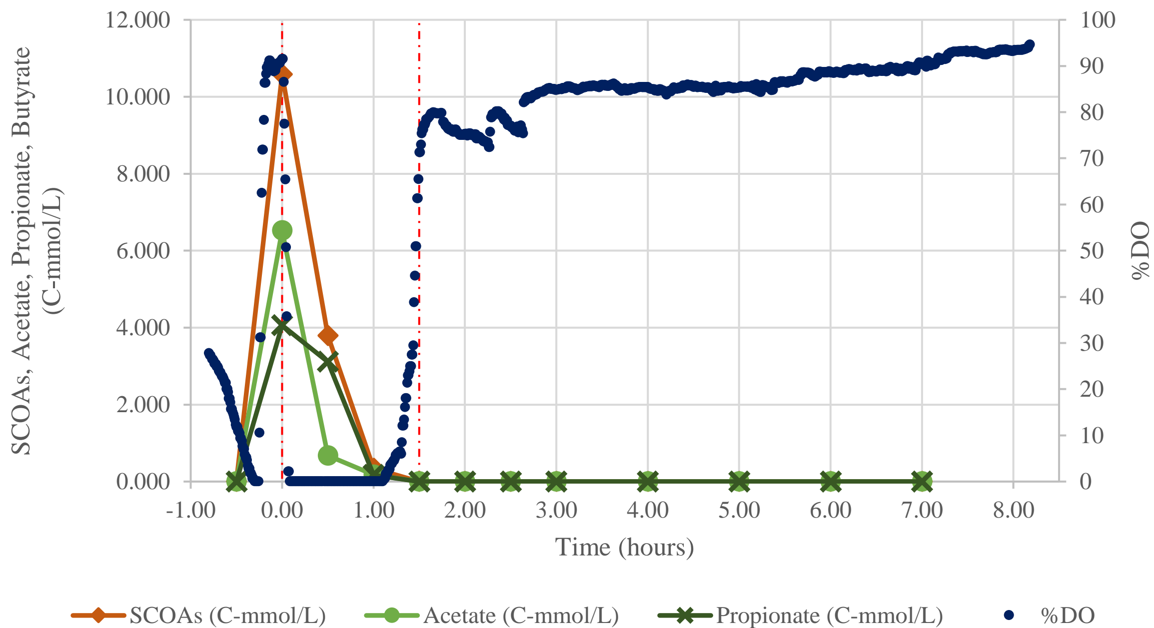

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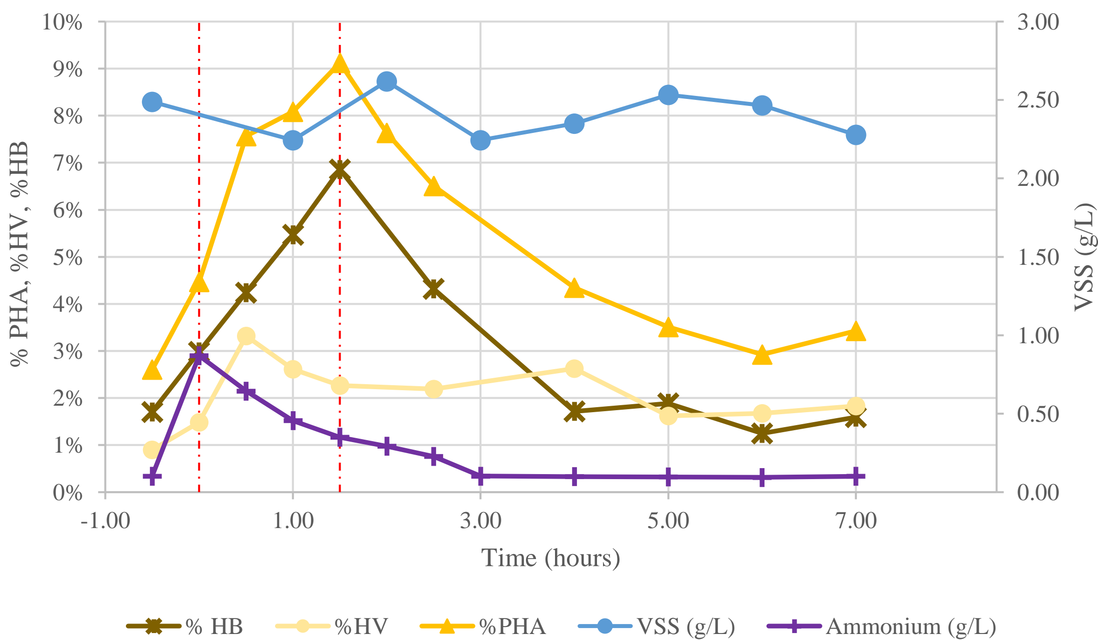

FIGURE 13 - Period 3 – 141st day of operation (12h cycle, 50 CmmolSCOAs/L, Stream A)

of 0.03 CmmolHA/L.h) and SCOAs were totally consumed, which all together clearly indicates the end of the feast phase and makes the F/F ratio for this cycle of 0.13.

Regarding the other monitored parameters, represented in Figure 13, the tendencies observed were similar to those of previous cycles. pH decreased from 8.55 to 8.15 after the feed and gradually increased to 8.51. Biomass concentration was on average 2.5 gVSS/L, with a maximum of 2.62 gVSS/L after the end of the feast phase. Lignosulfonates were not consumed during the monitored time and remained at about 4 g/L. Ammonium concentration decreased gradually since feeding and until 3 h.

4.2.2.4. PERIOD 4 – 161ST DAY OF OPERATION (12H CYCLE, 50 Cmmol SCOAS/L, STREAM B)

Finally in Figure 14 shows the SBR performance on the 161st day of operation. Corresponding to Period 4 of operation and, as said in 4.2., the reactor did not reach a PSS. SCOAs composition in the fed was 68.5/13.2/7.6/10.6 % of acetate, propionate, butyrate, and valerate. Only the first two are represented.

SCOAs variation during the cycle, represented in Figure 14 A, showed that acetate was also consumed at a higher rate (7.23 CmmolAcet/L.h) than propionate (3.60 CmmolProp/L.h). Acids depletion occurred at the same time, at 2 h. Regarding the PHA content and monomer composition, the results showed that the maximum content was achieved at 4 h for both monomers. With 6% cdw and a rate of 0.009 CmmolHB/L.h and 4% cdw and a rate of 0.021 CmmolHV/L.h for PHB and PHV, respectively. PHV production was different from previous cycles since the most significant accumulation occurred after 2.5 hours. Unlike other cycles the PHA production was not simultaneous to SCOAs consumption and its highest values were achieved after acetate and propionate depletion. This suggests consumption of other carbon sources that were not quantified.

pH and LS concentration had the same tendencies as previous cycles. The initial value of pH was 8.67, which decreased to 7.90 at 0 h and then slowly rose to 8.54. LS concentration was about 5 g/L with very small variations. Biomass (with a mean value of 3.1 gVSS/L) and ammonium concentration values throughout the cycle are coherent.

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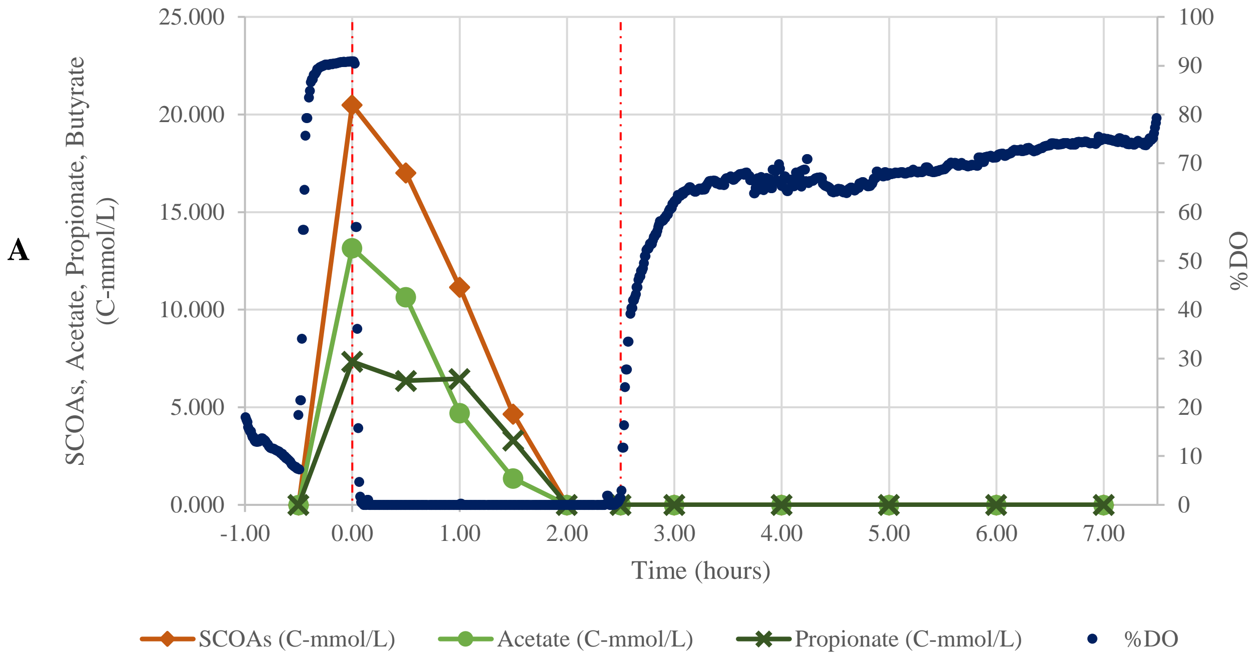

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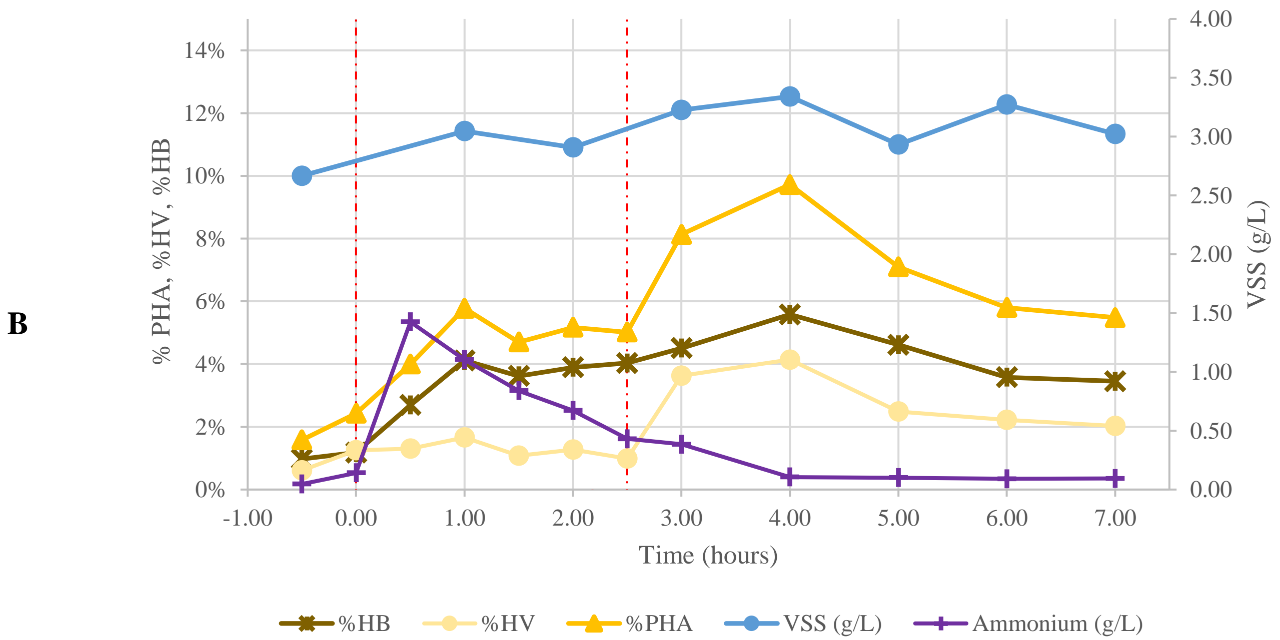

FIGURE 14 - Period 4 – 161st day of operation (12h cycle, 50 CmmolSCOAs/L, Stream B)

Biomass concentration reached its highest value (3.34 gVSS/L) at 4 h, the same time of ammonium exhaustion. All these parameters are represented in Figure 14 C.

Figure 14 also shows the overall variation of PHA content and SCOAs, as well as %DO profile is illustrated. SCOAs were consumed at a volumetric rate of 10.83 CmmolSCOAs/L.h and their exhaustion happened at similar time of %DO increase (between 1.5 and 2 h), which could indicate the end of the feast phase. However maximum PHA (10% cdw) was produced at a rate of 0.05 CmmolHA/L.h until 4 h, the same time biomass concentration was at its highest and ammonium finishes, also an indication of feast ending. From the experimental results, is was difficult to define the exact time of phase change. Considering %DO concentration, F/F ratio for this cycle was 0.22. Nonetheless it was evident by the data analysis that the culture was still not adjusted to the new substrate.

4.2.4. KINETIC AND STOICHIOMETRIC PARAMETERS

The kinetic and stoichiometric parameters obtained in each cycle are summarized in Table 7. Even though the cycles represented are from periods operated at different conditions many similar behaviours were observed. The general evolution of the main parameters was the same of works of MMC selection reported, namely Dionisi et al. (2006); Albuquerque et al. (2010); Oehmen et al. (2014), and that with HSSL as substrate, Queirós et al. (2014).

SCOAs were the only carbon sources consumed, and although there are other compounds liable to be consumed, like lignosulphonates, their complexity made them unlikely to be used. LS consumption was not verified in any cycle monitored, even though a slight use, possible by the microbial community without accumulation capacity, was reported in previous works with HSSL (Queirós et al. 2014). Nonetheless, a LS concentration increase, from 3 g/L to 5 g/L as a consequence of OLR increase, seemed to have no inhibitory effect on microorganisms as observed by Pereira et al. (2013) with a bioethanol producing yeast.

In all cycles, even if at different rates, there was a clear substrate preference for acetate over the other acids, like propionate and butyrate. This carbon source preference is consistent with literature reports (Lemos et al. 2006) and could be explained by the metabolic

pathways of the last two acids. Propionate can lead to the production of both PHB and PHV, to form the last propionate is converted to propionyl-CoA and condensate with acetyl-CoA. In order to obtain the necessary acetyl-CoA from propionate, propionyl-CoA must enter the TCA via methylmalonyl-CoA interconvertion to succinyl-CoA. Unlike acetate that directly originates acetyl-CoA, propionate consumption is dependent on the rate of decarboxylation of propopionyl-CoA, which can explain its lower rate. Butyrate also requires extra steps to be converted into PHB or acetyl-CoA, when compared to acetate, since acetyl-CoA is an important precursor for cell metabolism acetoacetyl-CoA cleavage to acetyl-CoA may be required (Fradinho et al. 2014). SCOAs volumetric consumption rates increased through time, which was an indication of the MMC was progressive adaptation to the substrate.

Polymer production was similar in the last three cycles presented. In the first, production rates were much higher than in cycles of 12h due to a particularly higher content of PHA achieved. As seen in 4.2.1.1., that is not necessarily and indicator of good selection. The values obtained for the specific production rate in the presented cycles were similar to those reported by Queirós et al. (2015), 0.03-0.04 CmmolHA/CmmolX.h, using the same substrate. Similar works also described polymer production rates of the same order of mangnitude, Albuquerque et al. (2010) achieved 0.05-0.011 CmmolHA/CmmolX.h with sugar molasses and Oehmen et al. (2014) had 0.04-0.28 CmmolHA/CmmolX.h with fermented molasses.

Productivities and YPHA/S yields decreased over the operational time, with the imposition of different parameters, which was expected. As said before, the goal of this step was to obtain a culture with good capacity to accumulate PHA and a biomass concentration as high as possible. The first period of operation successfully selected a PHA-storing population from the initial MMC, as seen by the high conversion of substrate into PHA and productivities obtained. However, the biomass concentration was low, so the OLR was increased to promote cell growth. This strategy was successful and resulted in the increase of the biomass concentration over time. On the other hand, because part of the substrate was diverted to growth, there was a decrease in the yields and productivities over time. Though this may seem a negative effect, because the culture was previously selected to eliminate

TABLE 7 - Kinetic and stoichiometric parameters obtained in the SBR cycles

Period	Day	F/F Ratio	X (g/L)	$-q_{SCOAs}$ (Cmmol/L.h)	$-q_{Acet}$ (Cmmol/L.h)	$-q_{Prop}$ (Cmmol/L.h)	$-q_{But}$ (Cmmol/L.h)	qp_{PHA} (CmmolPHA/L.h)	qp_{PHA} (CmmolPHA/CmmolX.h)	qp_{PHB} (CmmolHB/L.h)	qp_{PHV} (CmmolHV/L.h)	%PHA max	%PHB max	%PHV max	HB:HV	YPHA/S (CmmolPHA/CmmolS)	$Prod_{Esp}$ (gPHA/gX.h)	$Prod_{Vol}$ (gPHA/L.h)
P1	13	0.26	1	6.27	3.41	1.52	2.10	0.162	0.162	0.152	0.019	49%	36%	14%	64-36	76%	0.244	0.244
P2	48	0.08	2.4	10.08	9.55	2.66	3.98	0.072	0.030	0.045	0.027	15%	9%	6%	65-35	46%	0.148	0.354
P3	141	0.13	2.5	10.45	6.48	3.97	-	0.034	0.014	0.026	0.010	9%	7%	4%	63-37	55%	0.061	0.152
P4	161	0.22	3.1	10.83	7.23	3.60	-	0.049	0.016	0.009	0.021	10%	6%	4%	65-35	37%	0.024	0.075

non-accumulative microorganisms, there was no direct impact in the cultures accumulation performance in the last step of this work.

Although there was no pH control, in all cycles monitored the pH variation was under 1. With simple carbon sources wider pH variation were reported, 2.0 with acetate by Serafim et al. (2004), but when using real substrates some authors reported lower variations due to buffer capacities of the substract (Oehmen et al. 2014). With HSSL as carbon source, Queirós et al. (2015) and Queirós et al. (2014) had similar pH variations, 0.63 and 0,96, respectively) and also hypothesized a buffer behavior.

4.3. ACCUMULATION STEP

As said before, the accumulation is the final step of this process. After successfully obtaining an enriched culture in PHA-accumulating organisms in the selection process, the MMC should be submitted to an accumulation step in order to maximize the PHA production and achieve the highest volumetric production rate.

During the course of this project a total of nine accumulation assays, under batch conditions, were performed in order to better understand and optimize this step. The tests were made under different conditions, which are summarized in Table 5 of Chapter 3. The MMC selected in the previous step was collected from the SBR and inoculated to a batch reactor. The consecutive pulses of substrate were fed in order to potentiate PHA accumulation and avoid inhibition by substrate (Serafim et al. 2004). A new pulse was added after a sudden increase in the %DO in the reactor, indicating substrate depletion. Ideally the assay would end when the data collected online suggested that the MMC reached accumulation saturation, in particular when the OUR value was close to the endogenous OUR. However this was not possible in every cases due to volume and/or time constrains.

Two distinct sets of assays can be defined, the first with biomass from the PSS of P2 and the second from the PSS of P3 of the selection SBR (represented in Figure 9 of 4.2.1.). For all tests several kinetic and stoichiometric parameters were calculated and are presented in Table 9.

4.3.1. ACCUMULATION ASSAYS WITH BIOMASS FROM PERIOD 2

Once the SBR reactor achieved a PSS with sufficient biomass concentration, the first accumulation assays were performed. They aimed to understand the capacity of MMC to accumulate PHA using different carbon sources from the paper industry, in this case acidified HSSL (Stream A) and Condensate. These tests were also performed with and without presence of ammonium in order to optimize accumulation conditions. To understand the behavior of MMC to different SCOAs, assays using the HSSL matrix and synthetic acids (acetate and propionate, the major carbon sources in Stream A) were performed.

TABLE 9 - Kinetic and stoichiometric parameters obtained in the accumulation assays

Assay	Period	Substrate	Limitation	X (g/L)	_qSCOAs (CmmolSCOAs/ CmmolX.h)	qP (CmmolPHA/ CmmolX.h)	%PHA (max)	HB:HV	YPHA/S (CmmolPHA/ CmmolS)	ProdEsp (gPHA/gX.h)	ProdVol (gPHA/L.h)	Reference
AT1	P2	Stream A	None	1.2	11.76	0.18	45.2	77-23	0.10	0.17	0.20	This work
AT2	P2	Stream A	N	1.5	27.82	0.07	51.2	76-24	0.14	0.07	0.10	
AT3	P2	Condensate	None	2	3.74	0.23	43.2	100-0	0.24	0.10	0.21	
AT4	P2	Condensate	N	1.6	8.84	0.11	46.5	100-0	0.25	0.10	0.06	
AT5	P2	Matrix + Ac	None	1.9	11.63	0.13	43.6	100-0	0.14	0.15	0.28	
AT6	P2	Matrix + Prop	None	2.4	15.18	0.03	26.5	61-39	0.14	0.03	0.08	
AT7	P3	Stream A	N	4	42.56	0.07	74.4	77-23	0.20	0.07	0.27	
AT8	P3	Stream B	N	3.8	39.21	0.03	40.7	70-30	0.13	0.03	0.12	
AT9	P3	Condensate	N	1.7	40.46	0.01	9.6	100-0	0.02	0.01	0.02	
		HSSL	None	0.9	-	-	63.3	100-0	0.77 (gPHA/gCOD)	0.16	0.14	(Queirós et al. 2014)
		HSSL	None	3.2	-	0.09	4.6	80-20	0.12	0.01	0.02	(Queirós, Fonseca, et al. 2015)
		HSSL	N		-	-	34.6	76-24	0.79	0.07	-	(Rangel 2015)
		Fermented paper mill wastewater	N	-	-	-	76.8	86-14	0.76 (gPHA/gCOD)	0.15	0.08	(Jiang et al. 2012)
		Fermented sugar molasses	N	-	-	-	74.6	74-26 to 83-17	0.81	0.49	1.50	(Albuquerque et al., 2010)
		Fermented molasses	None	11.8	-	-	57.5	90-10	0.57-0.65	-	10.8	(Oehmen et al. 2014)
		Syntetic acetate	N	-	-	-	89	100-0	0.6	1.2	-	(Johnson et al. 2009)

4.3.1.1. STREAM A UNDER NO LIMITATION (AT1) AND UNDER N LIMITATION (AT2)

Two assays with Stream A were performed using biomass from the PSS of P2, one without limitations and other under ammonium limitation and are represented in Figure 15 and 15, respectively. In these tests five pulses of feeding with the same composition used in the SBR (SCOAs concentration of 35 Cmmol per pulse) were added to the batch reactor.

After each pulse, acids were consumed at different rates until exhaustion, and in both tests acetate had the highest volumetric consumption rate (34.3 and 8.1 CmmolAcet/L.h, the highest values from pulse 1), followed generally by propionate, butyrate and lactate. This was expected, since the same behavior was observed in the monitored cycles of the SBR.

Substrate consumption was accompanied by a gradual increase in the pH of the reactor and its depletion led to a sudden increase of %DO, as expected. When this pattern in pH and %DO was observed another pulse was added because it indicated substrate exhaustion.

In both cases OUR became closer to the endogenous OUR over time. Since endogenous OUR corresponds to the oxygen needed for cell maintenance, the lowering of the oxygen demands could be an indication that the culture was not metabolizing the carbon source for PHA accumulation. This consequentially means that the culture achieved the maximum storage capacity. Such was further demonstrated by the decrease of consumption rate of SCOAs in each pulse, and by the PHA content.

For the assay without limitation, the maximum PHA content obtained was 45.2% cdw, with a specific production rate of 0.18 CmmolPHA/CmmolX.h. The overall yield $Y_{PHA/S}$ obtained in this accumulation test was 0.10 CmmolPHA/CmmolS. The monomeric composition of the copolymer was uniform throughout the test and by the end of the assay was of 77:23 (HB:HV).

For the assay with ammonium limitation, the maximum PHA accumulation was 51.2% cdw, with a production rate of 0.18 CmmolPHA/CmmolX.h, and the overall yield $Y_{PHA/S}$ obtained in this accumulation test was 0.14 CmmolPHA/CmmolS. The monomeric composition of the copolymer was of 76:24 (HB:HV) and was uniform throughout the test.

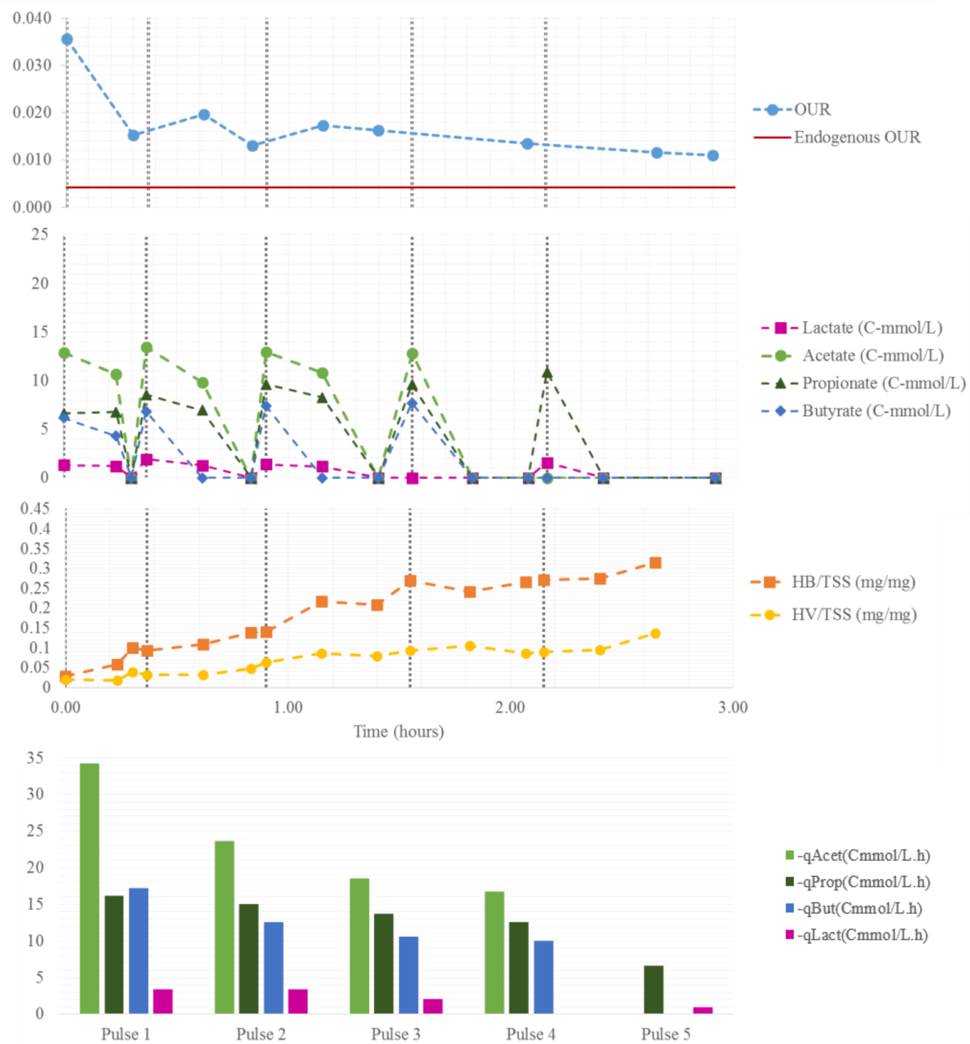

FIGURE 15 – AT1: Accumulation assay Stream A, under no limitation, P2

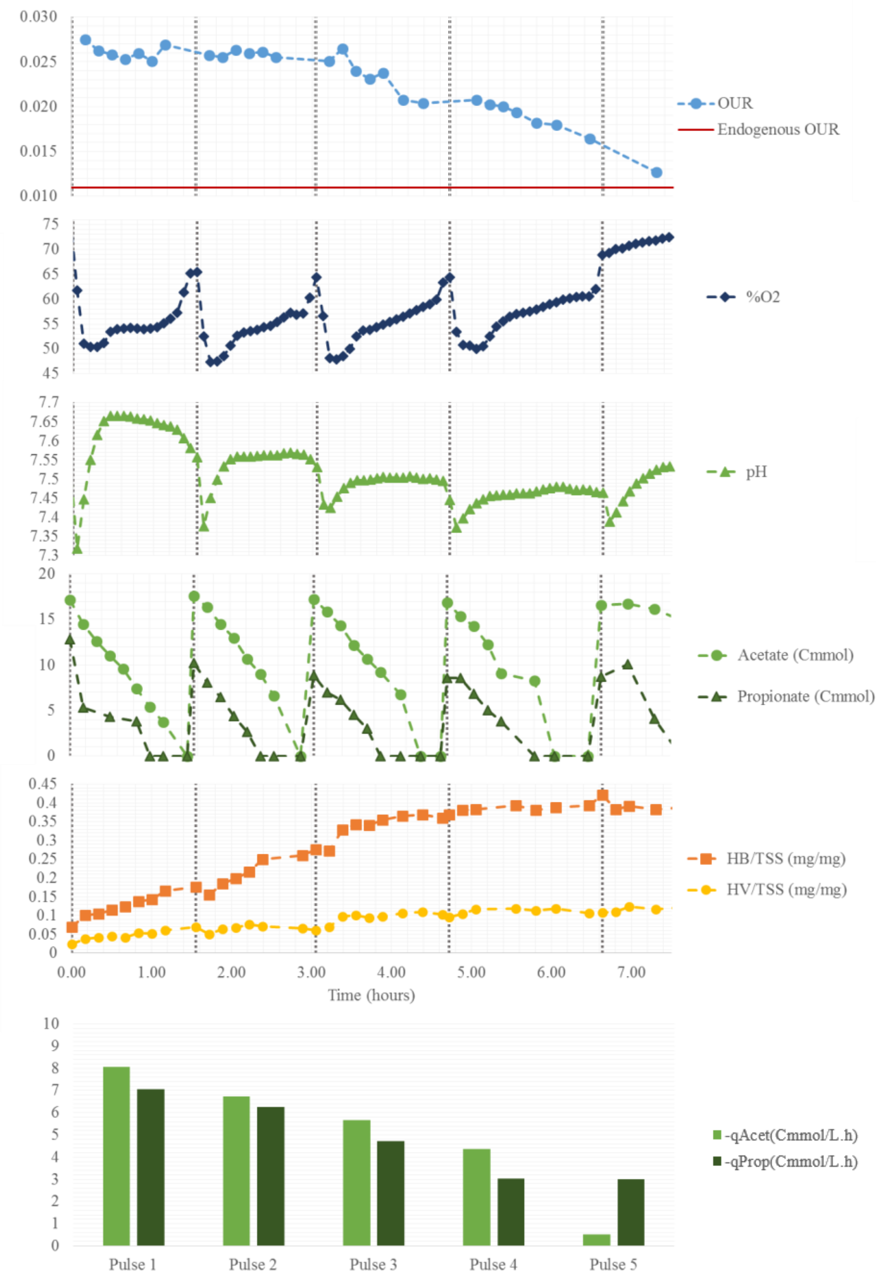

FIGURE 16 – AT2: Accumulation assay Stream A, under ammonium limitation, P2

The polymer composition was very similar in both tests since the substrate was the same.

Comparing the two assays, although in the assay without limitation the same amount of substrate was consumed in a shorter period of time, a higher PHA content was obtained in the assay without ammonium. Since ammonium limitation has a direct effect in cell growth, in this condition carbon flow is redirected to PHA synthesis. Consequently a better result was expected and verified in AT2, with more 6% cdw of polymer stored and a higher $Y_{PHA/S}$ yield. The lower results for AT1 could be explained with an increase of biomass concentration. However this parameter was only determined for the beginning of the assay and this hypothesis could not be verified.

4.3.1.2. CONDENSATE UNDER NO LIMITATION (AT3) AND UNDER AMMONIUM LIMITATION (AT4)

One of the goals of this work was to integrate different by-products from Caima in a three stage PHA production process, namely HSSL and Condensate. Since the main carbon source in Condensate is acetate, this substrate was only used in the last stage of the process. It was expected that the culture selected using a mixture of SCOAs would be able to use only acetate to accumulate PHA. Nonetheless, Condensate has in its composition other potential inhibitors (such as methanol and furfural) to which the culture was not adapted and that could undermine the accumulation process.

To understand the enriched MMC response to this substrate two assays were conducted, using biomass from the PSS of P2 and Condensate (diluted in order to have 35 CmmolAcetate in each pulse), with and without ammonium. However, since the acetate content of the Condensate was determined previously to its storage some degradation must have occurred since the acetate concentration of each pulse posteriorly detected was around 20-25 CmmolAcetate. These assays are represented in Figures 17 and 18.

Generally the tendencies observed in the assays with Stream A were the same with Condensate. After each pulse, acetate was gradually consumed and pH increased, at the time of substrate depletion an increase in the %DO was observed. Over time, OUR decreased to

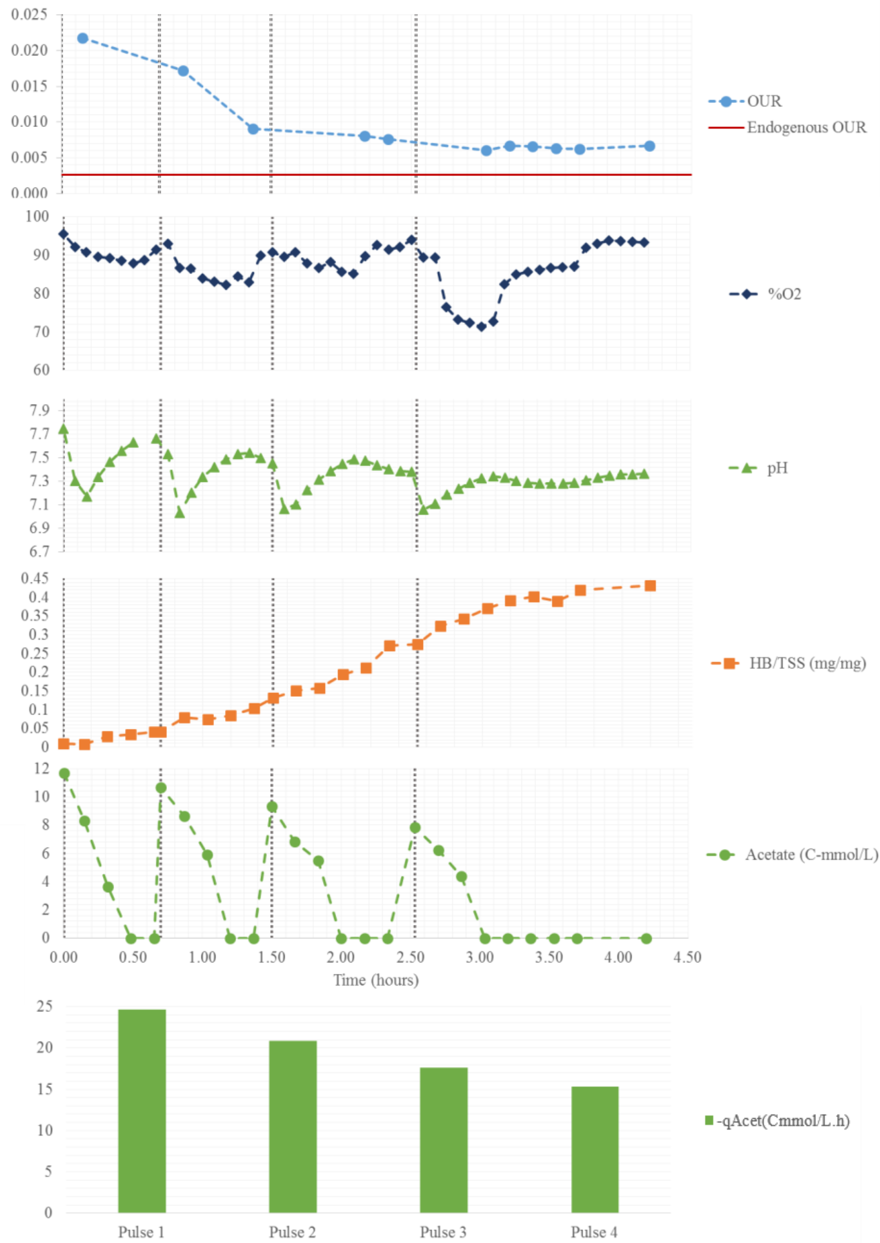

FIGURE 17 – AT3: Accumulation assay Condensate, under no limitation, P2

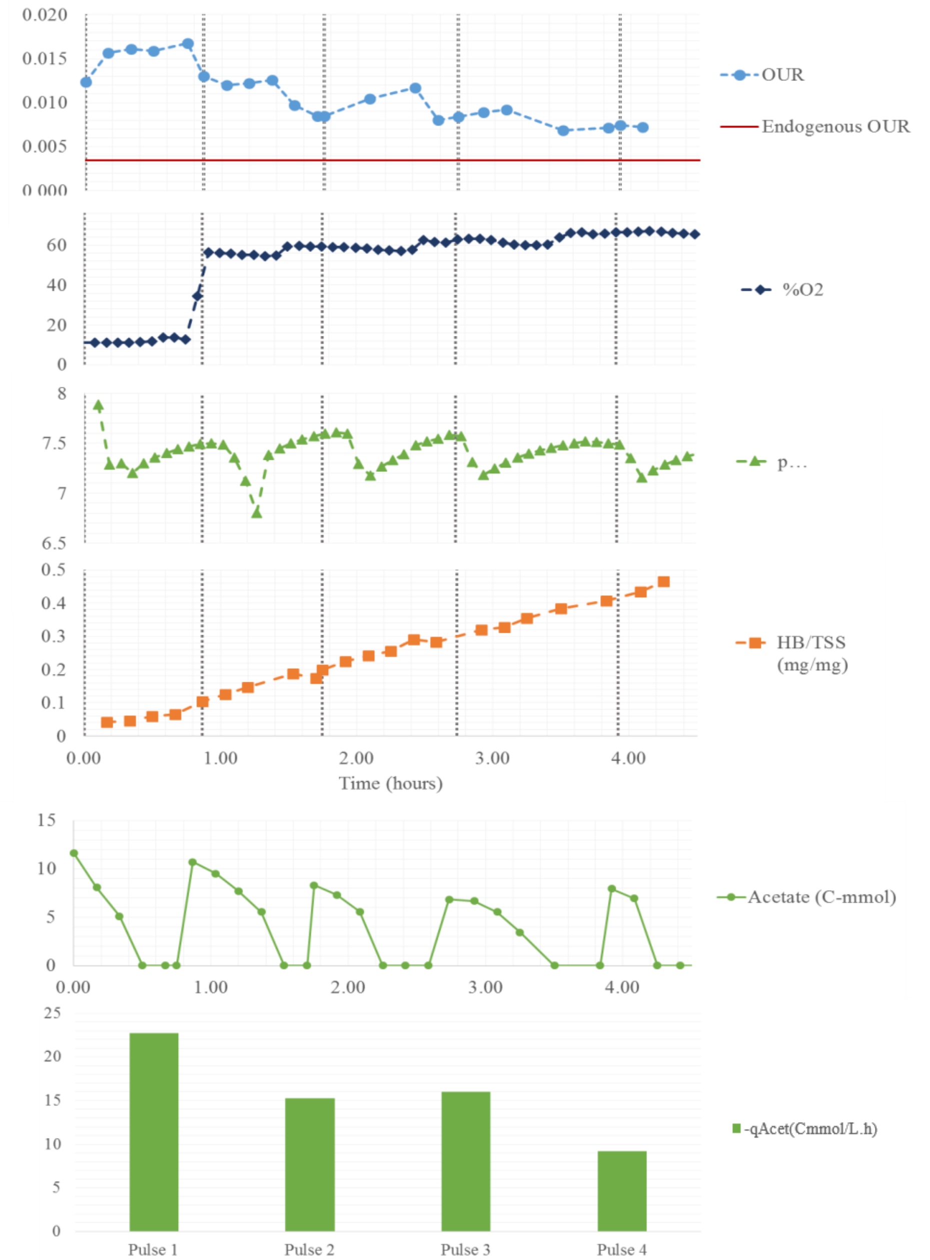

FIGURE 18 – AT4: Accumulation assay Condensate, under ammonium limitation, P2

values close to the endogenous value and acetate consumption rates decreased after each pulse. Acetate consumption in both cases was very similar, 24.7 and 22.7. CmmolAcetate/L.h, for the assays without and with limitation. The volumetric consumption rates decreased over time in both cases, but more rapidly for the test under N limitation

Generally the tendencies observed in the assays with Stream A were the same with Condensate. After each pulse, acetate was gradually consumed and pH increased, at the time of substrate depletion an increase in the %DO was observed. Over time, OUR decreased to values close to the endogenous value and acetate consumption rates decreased after each pulse. Acetate consumption in both cases was very similar, 24.7 and 22.7 CmmolAcetate/L.h, for the assays without and with limitation. The volumetric consumption rates decrease over time in both cases, but more rapidly for the test under N limitation.

For the assay without limitation, the maximum PHA content obtained was 43.2% cdw and the overall yield obtained in this accumulation test was 0.24 CmmolPHA/CmmolS. The test under ammonium limiting conditions achieved a maximum polymer content of 46.5% cdw and a yield of $Y_{PHA/S}$ of 0.25 CmmolPHA/CmmolS. As expected, since the only SCOA present was acetate, a homopolymer of HB was produced in both cases.

It is difficult to draw concrete conclusions on the effect of ammonium limitation because, due to operational volume limitations, it was not possible to add another pulse to AT3. Nonetheless considering the time before the addition of the fifth pulse in AT4, it was possible to see that the assay without limitation had a slightly better performance. The accumulated polymer was 42.0% cdw for the assay without limitation with a PHA production rate of 0.23 CmmolPHA/CmmolX.h, while for the assay without N the accumulated polymer before pulse 5 was 40.7% cdw with a production rate of 0.11 CmmolPHA/CmmolX.h.

4.3.1.3. MATRIX WITH ACETATE (AT5) AND PROPIONATE (AT6) UNDER NO LIMITATION

In order to understand how the culture was adjusted to each substrate, accumulation assays using synthetic acids and the HSSL matrix were performed. Acetate and propionate

were tested since they were the main SCOAs produced in the acidification process. Each acid was tested separately by the addiction of 35 Cmmol of acid per pulse to a batch reactor

The assay with acetate, Figure 19, demonstrates that, as seen in previous tests, the MMC was well adapted to the consumption of this acid that occurred at high rates for every pulse. The behavior was very similar to the assays with Stream A and Condensate, but the rise of the %DO was much more evident. This can be explained by the fact that there is only one SCOA being consumed, unlike with Stream A, and there are no inhibitors present other than those in which the culture was selected, unlike with Condensate. Besides, with acetate being the only carbon source present, a quicker accumulation was expected since the consumption rate for this SCOA was always the highest in both the selection SBR and the previous accumulation assays.

For these reasons the performance in this assay was better than in previous tests with a maximum accumulation of PHB of 43.6 % and a yield of 0.14 CmmolPHA/CmmolS, in only 3.6 h. The results also indicate that the culture could probably still accumulate more polymer since the OUR had not reached the endogenous value and the consumption rates were still very high. Therefore the test should have been continued, which was not possible because the reactor had already reached its maximum volume ate the end of the fifth pulse.

Regarding the assay with propionate, Figure 20, it is clear that the MMC had a lower preference for this acid when compared with acetate. Which was expected since the acetate preference was already verified in both accumulation tests and SBR cycles conducted before.

The data collected however showed that the MMC seemed to adapt to the carbon source over time, consuming it faster at each pulse. In fact, OUR had the opposite tendency in this test than what was observed in previous ones, increasing over time as a sign of higher substrate metabolization. Some works have already focused on the behavior of MMC submitted to a substrate shift and their substrate preferences (Lemos et al. 2006; Albuquerque et al. 2012; Carvalho et al. 2014). Lemos et al. (2006) selected two separated cultures with acetate and propionate and then feed them with the other SCOA and a mixture of both. They observed that the culture was capable to accumulate PHA, even if in some cases the SCOAs uptake rates were inferior. They also observed that

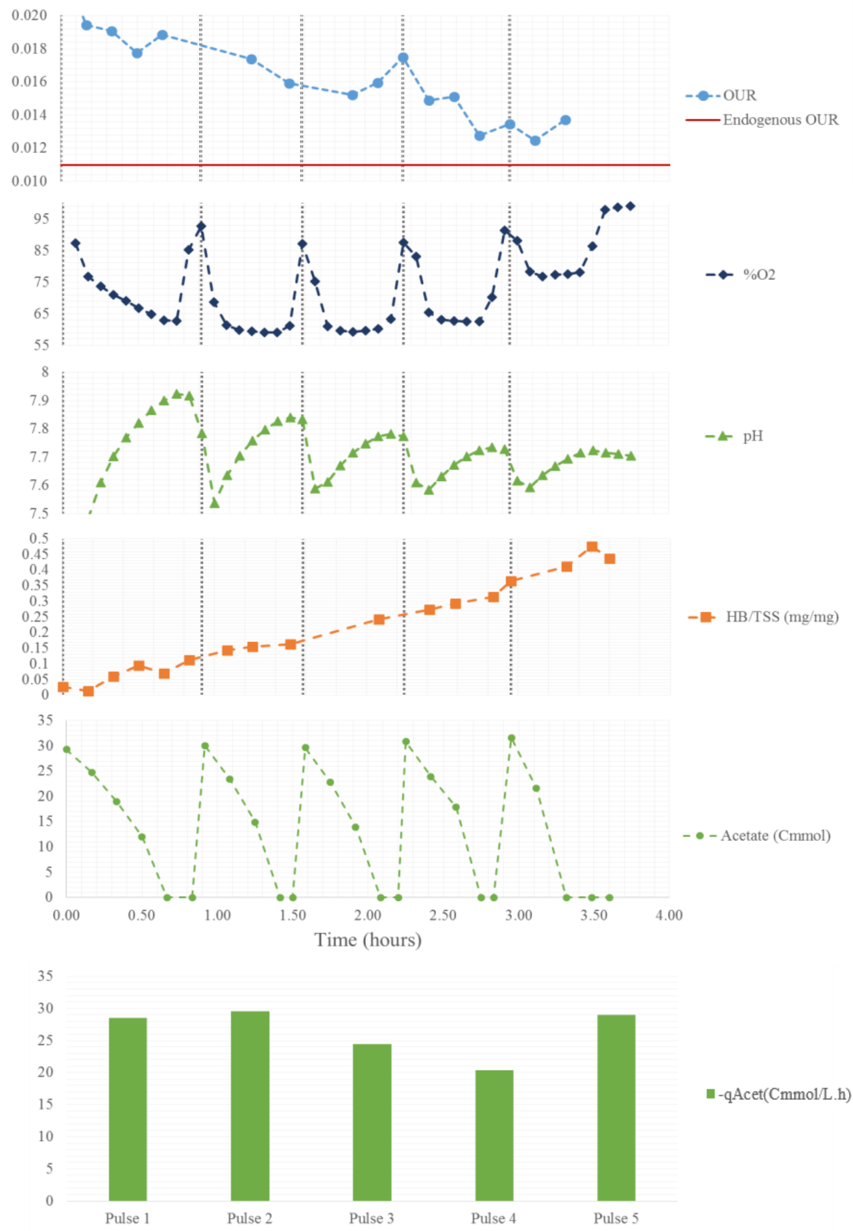

FIGURE 19 – AT5: Accumulation assay Matrix + Acetate under no limitation, P2

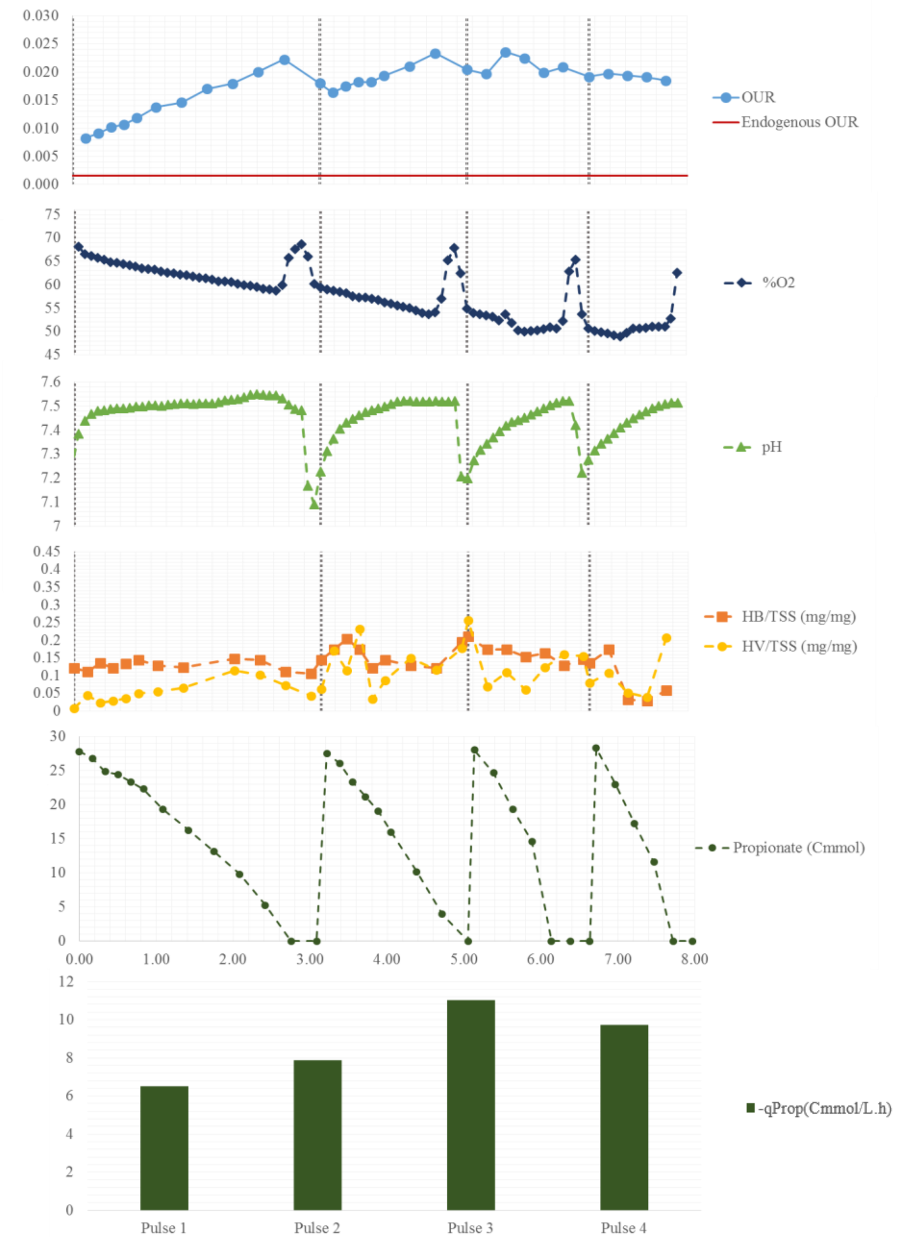

FIGURE 20 – AT6: Accumulation assay Matrix + Propionate under no limitation, P2

cultures selected with different substrate sources produced different copolymers when feed the same SCOAs mixture.

Even though substrate intake improved during the test, this was not translated into an increase in accumulation over time. In fact PHA content was very inconsistent during the assay and the maximum PHA produced, 26.5%, was very low in comparison with the other assays (Table 9). The low accumulation can be explained by the fact that only a fraction of microorganisms can use propionate for their metabolic pathways. Propionate as the only substrate source could lead to more competition in the microbial community and reduce substrate utilization efficiency (Wang et al. 2013).

Since propionate was the precursor for PHA biosynthesis in this assay two monomers were produced, as expected. Propionate can follow two metabolic pathways one originating PHB and other PHV (as illustrated in Figure 4 of Chapter 2), and so the polymer produced was an HB:HV mixture with and average proportion of 61:39.

4.3.2. ACCUMULATION ASSAYS WITH BIOMASS FROM PERIOD 3

After insuring a good storage response, the SBR operated in order to increase biomass for this would have a positive impact in the volumetric productivity of the accumulation step. For this reason when the MMC of the selection SBR reached a PSS in P3, new accumulation assays were performed. In this set of assays, three main substrates were tested: Stream A, Stream B and Condensate.

Since these assays used biomass from P3 the initial concentration of biomass was higher and the amount of SCOAs in each pulse was 50 Cmmol in order to mimic the conditions of the selection SBR. Tests were conducted under ammonium limitation since better PHA contents were achieved in these conditions in tests from P2. Since ammonium limitation influences cell growth by reducing enzymatic activity, it is expected that the carbon flow is redirected towards PHA synthesis (Cavaillé et al. 2013). Moreover literature as showed that this limitation promotes accumulation in processes using real substrates (Mengmeng et al. 2009) and HSSL, in particular (Rangel 2015).

Finally, to overcome some of the difficulties of previous tests, the feeding pulses were concentrated so that more pulses could be added before the volume limit of the

reactor was reached. In this way, accumulation assays could have been prolonged in order to reach saturation and achieved the highest PHA content possible.

4.3.2.1. STREAM A UNDER AMMONIUM LIMITATION (AT7)

Overall the same tendencies of AT1 (Figure 15) were observed in AT7 as expected, since the substrate was the same (Figure 21). After each pulse, the SCOAs were consumed until exhaustion, which was coincident with pH and %DO increase. By acids depletion, a sudden increase in the oxygen concentration was observed and indicated that another pulse could be added.

In this accumulation assay, 9 pulses were added and it was possible to reach a saturation state, which did not occurred in AT1. A saturation point can be inferred by the analysis of several parameters. PHA content in biomass increased slowly until a maximum of 74.4% cdw at 11 h. After this time the polymer content in the cells slightly decreased to 61% cdw by the end of the test. SCOAs consumption rates also indicated saturation since they rapidly decreased until the fifth pulse and after that they remained fairly low and constant. Finally the MMC saturation can also be observed in the OUR variation, the OUR measured decreased over time until 7 h and after that it remained constant and very close to the endogenous OUR value calculated.

This test had the better performance of all the accumulation assays conducted in this work and also of all of those using HSSL as carbon source. This test achieved a maximum PHA content over 70%, with a monomer composition of 77:23 of HB:HV. Production yields was of 0.20 CmmolPHA/CmmolS and the production rate was of 0.07 (CmmolPHA/CmmolX.h).

4.3.2.2. STREAM B UNDER AMMONIUM LIMITATION (AT8)

In the assay using Stream B, AT8 (Figure 22), the MMC had a very similar response to AT7 with Stream A. As said before, the main difference between this two

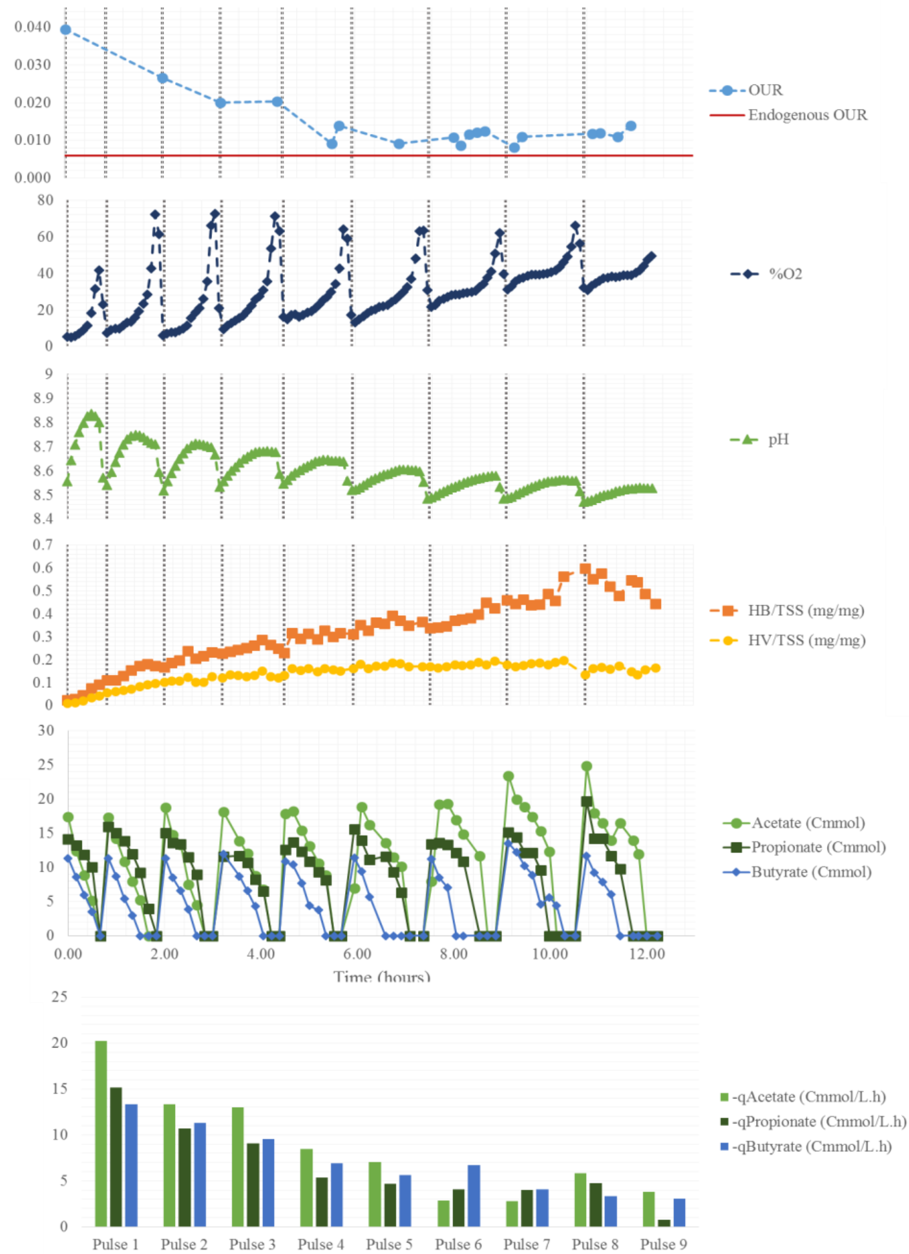

FIGURE 21 – AT7: Accumulation assay Stream A, under N limitation, P3

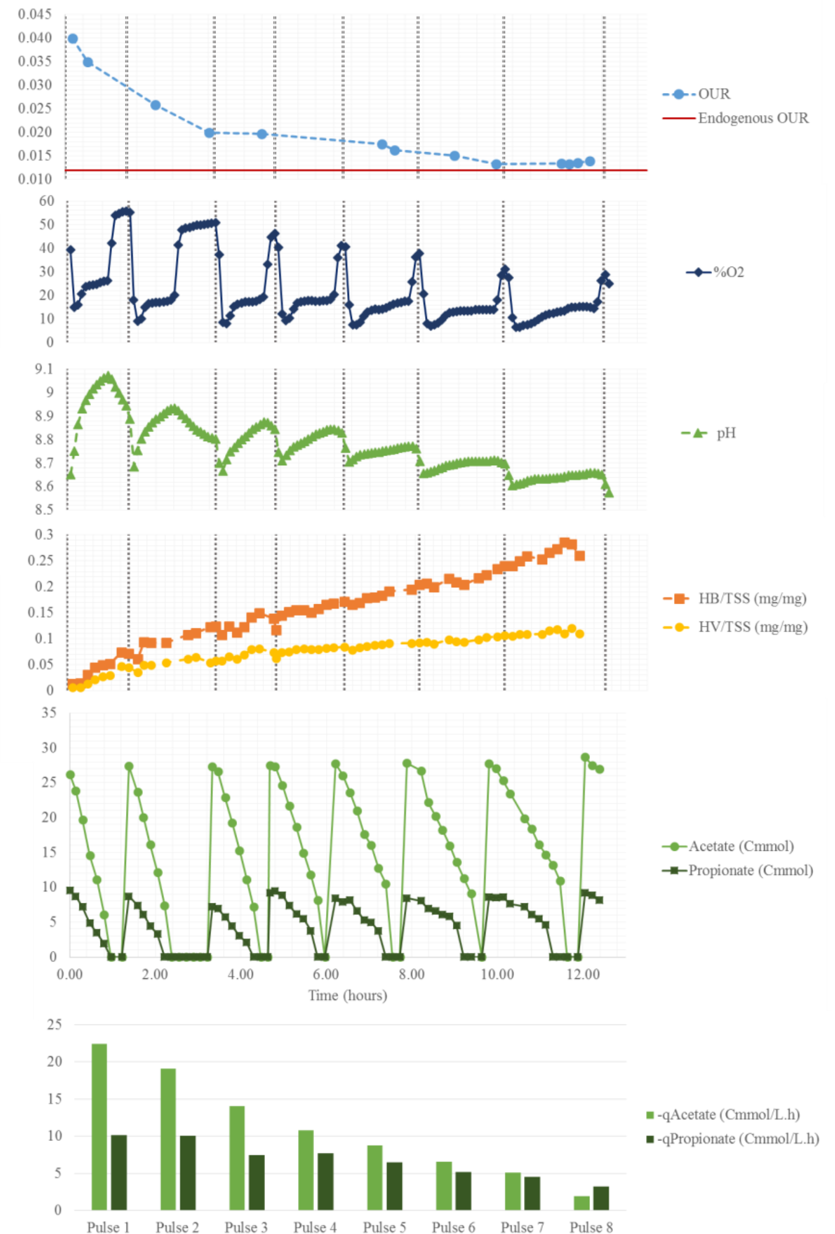

FIGURE 22 – AT8: Accumulation assay Stream B, under N limitation, P3

streams was the relative SCOAs composition. Although the culture was not acclimatized to this substrate, significant inhibitory effects were not expected.

Eight pulses with 50 Cmmol of SCOAs were added and the performance was very similar to what was described in AT7. Nonetheless, some aspects can be highlighted, two distinct increases in %DO can be observed in each pulse. A lower one after the addition of the pulse and another latter and more evident corresponding to the SCOAs exhaustion.

This assay also seemed to reach saturation since the OUR measured was constant and after 9.6 h was very close to the endogenous, besides this the consumption rates were very low after the 7th pulse.

The main difference of the use of Stream B was the monomer composition of the PHA produced, with 70:30 of HB:HV. This test produced the copolymer with the highest HV content. It showed that the variation of the conditions of acidification in the first step of the process can lead to the manipulation of the PHA polymer produced in the last step.

The production rates and yields were close to those observed in previous tests, production yields was 0.13 CmmolPHA/CmmolS and the production rate was of 0.03 (CmmolPHA/CmmolX.h).

4.3.2.3. CONDENSATE UNDER AMMONIUM LIMITATION (AT9)

The final assay conducted was fed with Condensate in pulses with 50 Cmmol of acetate (Figure 23). In the previous test with this substrate (Figures 17 and 18), the MMC presented significant accumulations and production ratios and these tests showed room for improvement since the culture apparently had not reached polymer saturation. Based on this, more concentrated pulses were added in the assay.

Even though the test followed the same tendencies as the previous one, it achieved a lower PHA content. The variations in %DO were less evident but acetate was completely consumed in every pulse. The consumption rates calculated for this test were similar to those achieved in the other assays with Condensate. However the substrate was not being directed

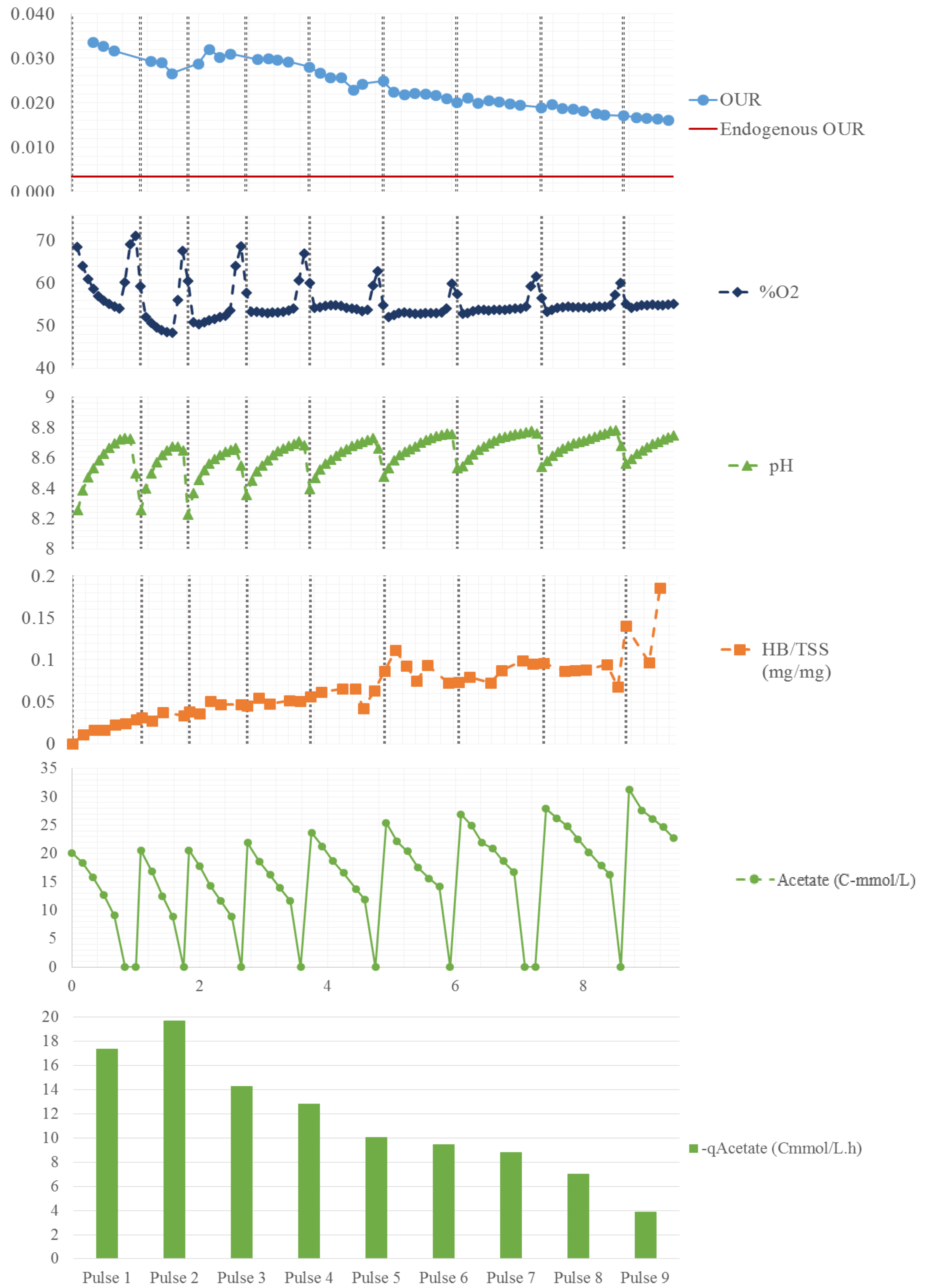

FIGURE 23 – AT9: Accumulation assay Condensate, under N limitation, P3

towards PHB production, since the values of polymer content in the cells were the lowest of all assays.

By the end of this test the culture had accumulated less than 20% of PHB, with a production rate of 0.01 CmmolPHA/CmmolX.h) and a yield of 0.10 CmmolPHA/CmmolS. The OUR was still far from the endogenous value, as expected since the culture was nowhere near saturation.

This results were not expected since the use of Condensate as substrate for PHA production using the selected MMC was already validated in assays AT3 and AT4 and although the biomass had a different origin (in this case it was from Period 3 of the SBR operation), the same culture had already showed good accumulation capacity in tests AT6 and AT7. The most likely explanation for the obtained results is the inhibitory effect of the compounds present in the Condensate. In order to enable the addition of more pulses but maintaining the same acetate quantity the pulses were concentrated in these assays (200 mL were added instead of 500 mL). This change concentrated not only the carbon source but also all the inhibitory compounds present in the Condensate, namely methanol and furfural. In high concentrations, literature shows that these compounds led to PHA production inhibition (Pan et al. 2012; Dietrich et al. 2013).

4.3.3. OVERALL PERFORMANCE

Table 9 resumes the kinetic and stoichiometric parameters calculated for each accumulation assay as well as some other relevant works in the area. Overall the results obtained in the accumulation tests go accordingly to what was expected.

The increase of the biomass concentration of the SBR led to a higher concentration on the accumulation assays which had a direct impact in the PHA volumetric production, usually a limiting aspect of working with MMC (Oehmen et al. 2014).

Ammonium limitation was, as stated before, an important parameter in the accumulation process since it limits cell growth and carbon flow is diverted towards PHA production. This phenomenon was evident in the assays conducted, since tests with N limitation had higher SCOAs consumption rates and PHA cell contents.

Finally, the culture was able to accumulate PHA with all substrates fed, even those with different composition than the one it was selected in. This shows the importance of the selection with a mixture of SCOAs, as mixed-carbon-acclimated cultures are quicker to adjust to substrate variations (Wang et al. 2013). Microorganisms have different metabolic pathways that allow for the use of different carbon sources, the enrichment of the culture in a SCOAs mixture leads to a diverse microbial composition and ensures better substrate utilization efficiency for every SCOA fed (Albuquerque et al. 2012).

Good results were achieved using each substrate source proposed in the beginning of this work. The selected MMC accumulated a maximum of 74.4% cdw of PHA copolymer in the cdw using Stream A with a volumetric productivity of 0.27 gPHA/L.h and a YPHA/S yield of 0.20 CmmolPHA/CmmolS (AT7). Stream B had a different SCOA profile which lead to the production of the copolymer with the highest HV content. A maximum PHA of 40.7% cdw was obtained in AT8 with a volumetric production of 0.12 gPHA/L.h and an YPHA/S yield of 0.13 CmmolPHA/CmmolS. Finally, Condensate led to the production of PHB with a maximum of 43.2% cdw and a volumetric production of 0.21 gPHA/L.h and an YPHA/S yield of 0.24 CmmolPHA/CmmolS.

As expected Stream A was the substrate that led to the best result since it was the one in which the culture was selected and acclimatized. Both the maximum polymer content and the volumetric productivity of the AT7, were very promising results when comparing with what was already achieved in previous works with HSSL. As seen in Table 9, other works with HSSL as a substrate for PHA production had lower results compared to this work. Queirós et al. (2014) obtained the highest polymer content - 63.3% cdw, 11% less than AT7 - and volumetric production - 0.14 gPHA/L.h, almost half than what was achieved in this work, mainly due to the higher biomass concentration in the assay. However both Queirós et al. (2014) and Rangel (2015) had higher YPHA/S. This work was also the first were the acidification step occurred separately, and its manipulation allowed for the tailoring of the copolymer produced. In this case, it was possible to produce both and PHB monopolymer and an P(HB-co-HV) copolymer, with an HV content ranging from 25 to 39%.

The best polymer content achieved with MMC, of 89% cdw, was reported by Johnson et al. (2009) using acetate as feed in the accumulation process. However, considering the best results obtained in this work, they are comparable to those obtained in other works using

MMC and complex substrates. Jiang et al. (2012) used fermented paper mill wastewater as carbon source and produced a copolymer of P(HB-co-HV), with a maximum accumulation of 76.8% cdw and a volumetric productivity of 0.08 gPHA/L.h. Using fermented sugar molasses, Albuquerque et al. (2010) reported a maximum accumulation of 74.6% cdw and a volumetric productivity of 1.50 gPHA/L.h. Oehmen et al. (2014) reported one of the highest volumetric productivities with MMC and real substrates, 10.8 gPHA/L.h with fermented molasses due to an high biomass concentration, 11.8 g/L . These results show that although the polymer content achieved in this work was high according to literature, there is still room for improvement regarding the volumetric productivity. This can be accomplish by maintaining the strategy of OLR increase in the SBR selection reactor.

As Table 9 shows this work has some of the lowest yields of YPHA/S compared to other works in the area and to the values obtained in the selection step. This may be explained by the complexity and toxicity of the HSSL. In these assays the OLR fed to the reactor was much higher than in the selection step and while SCOAs were being consumed other compounds concentrations were accumulating with each pulse. Because of the presence of phenolic compounds of low molecular weight in the medium that could diffuse to the interior of the cell, some of the carbon could be diverted to energy production to maintain intracellular stability (Pereira et al. 2012) or even to the production of extracellular polymeric substances (EPS) to protect itself from the medium (Poli et al. 2011).

CHAPTER 5: CONCLUSION

This work focused on the optimization of the selection and accumulation of a three-step process for PHA production using HSSL, a by-product of the pulp industry, as substrate. In the first step, which was not the objective of this work, HSSL was submitted to acidogenic fermentation in different conditions and originated two distinct streams of effluent with distinct SCOAs composition that were used in the subsequent steps.

The second step consisted in the selection of a MMC enriched for PHA accumulation. To achieve this goal a SBR was operated during 180 days, under ADF conditions. Three pseudo-stationary states were reached after successive increases in the selective pressure by the decrease of the cycle duration and the increase of the OLR fed. This was a clear indication that the culture was able to adapt to the carbon source and achieve a stable PHA production. The strategy used successfully met the two main goals for the selection step: an MMC with increasingly good PHA storage capacity and biomass concentration. The increase of the OLR proved to be an efficient approach to increase cell density and no inhibitory effects were observed.

In the third step the selected MMC was submitted to several accumulation assays under batch conditions. The culture showcased a good accumulation capacity indicating that the selection process was well performed. The best accumulation in this work reached a maximum content of PHA of 74.4% cdw and a volumetric productivity of 0.27 gPHA/L.h, the highest values reported using HSSL. The manipulation of the SCOAs profile of the stream used as carbon source in this last step led to the production of a P(HB-co-HV) copolymer with an HV content variation of 7%. The last step of this work also showed that an MMC selected using acidified HSSL with a particular SCOAs composition can be used in accumulation processes using different substrates with SCOAs as a main carbon source. In this case HSSL acidified under different conditions and Condensate.

This work helped to show that while mixed cultures cannot compete with pure cultures in terms of PHA production productivities they can be major players in the valorization and treatment of low-value substrates. The MMC capacity to adjust to the operational conditions and variations of the process ensures the success of its use in systems

as dynamic as the ones using complex substrates: in this case using by-products of the pulp industry, HSSL and Condensate. These are currently sold to produce new chemicals or burned for energy production. The big amounts of released of such by-products could be the starting point to their inclusion in a biorefinery concept with PHA production as the potential to be a more economically and ecologically sustainable solution.

CHAPTER 6: FUTHER WORK

The study of the valorization of byproducts of the pulp and paper industry through PHA production is not finished with this work. It is important to continue the optimization of all the steps mentioned in this work, and to understand how the different operational conditions (SRT, HRT), pH, temperature, cycle length, OLR, influent substrate concentration and nutrient concentration can influence the process. Regarding the acidification step in particular, it is central to understand how its manipulation can lead to different SCOAs profiles, since it greatly influences the monomer composition of the polymer in the end of the process.

Another important aspect would be the characterization of the microbial community along the selection step. Several samples were collected throughout the system's operating period. However, due to time constrains, it was not possible identify the different groups of microorganisms and relate them with the storage capacity and kinetics of the MMC.

A more exhaustive study of the properties and characteristics of the polymers produced is another interesting work since it will allow to understand and propose possible application for each PHA obtained. In particular its thermal properties, through thermogravimetric analysis, and mechanical properties, to determine the tensile strength, Young's modulus of elasticity and viscoelastic behavior. Finally, the study of the microstructures of the PHA polymer chains would also be a relevant work. In order to understand of how homopolymers, random copolymers, block copolymers, block-random copolymers can impact the final applications of PHA.

As a final point, the integration of the process described in the work in a biorefinary basin in the HSSL should continue to be evaluated. As well as the possible inclusion of other byproducts and bioprocesses in order to achieved a more efficient and comprehensive process.

CHAPTER 7: REFERENCES

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